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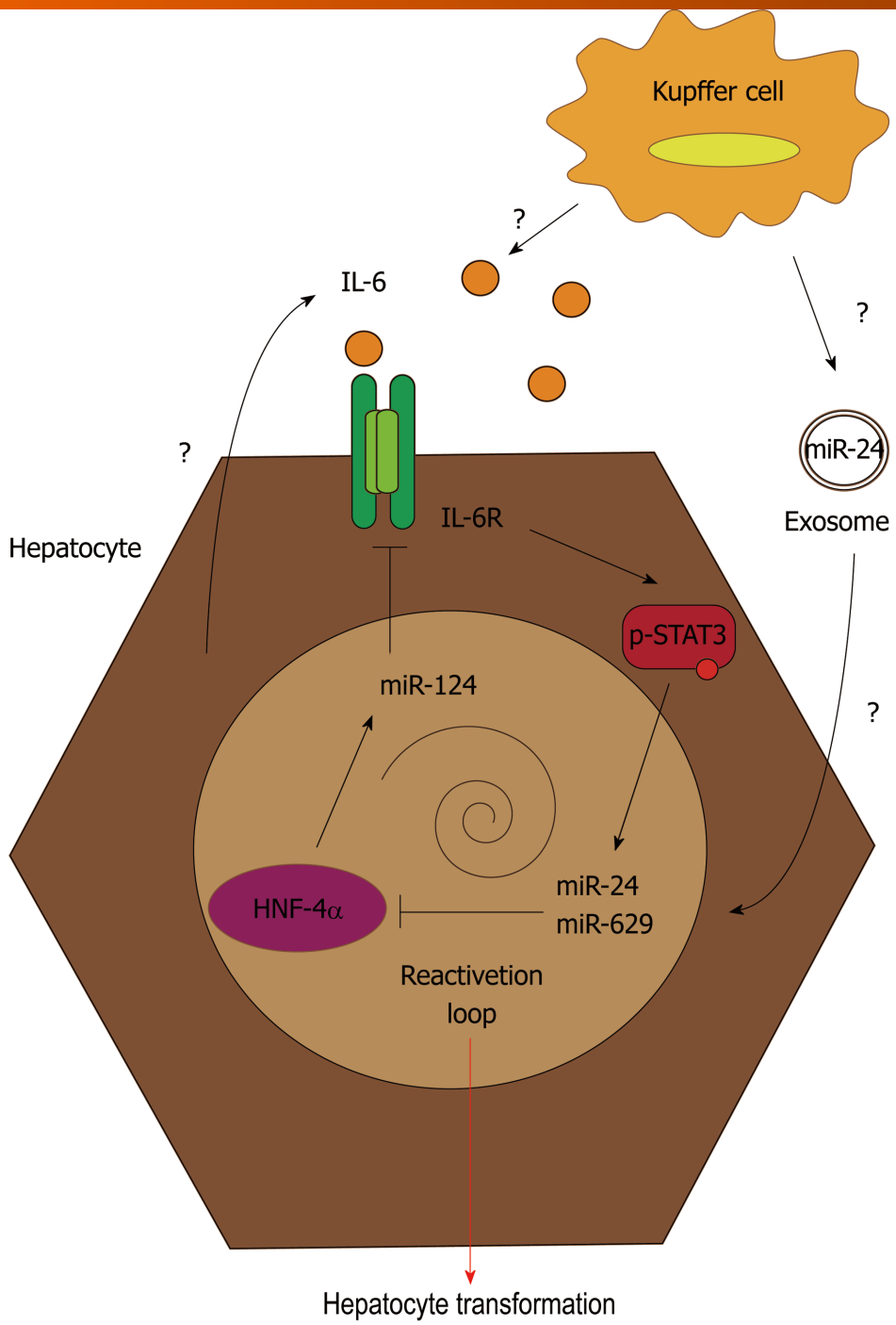

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EDITORIAL

Advanced endoscopic technologies for colorectal cancer screening

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Abstract

Colorectal cancer is the third most common cancer in men and the second most common cancer in women worldwide. Diagnosing colorectal has been increasingly successful due to advances in technology. Flexible endoscopy is considered to be an effective method for early diagnosis and treatment of gastrointestinal cancer, making it a popular choice for screening programs. However, millions of people who may benefit from endoscopic colorectal cancer screening fail to have the procedure performed. Main reasons include psychological barriers due to the indignity of the procedure, fear of procedure related pain, bowel preparation discomfort, and potential need for sedation. Therefore, an urgent need for new technologies addressing these issues clearly exists. In this review, we discuss a set of advanced endoscopic technologies for colorectal cancer screening that are either already available or close to clinical trial. In particular, we focus on visual-inspection-only advanced flexible colonoscopes, interventional colonoscopes with alternative propulsion mechanisms, wireless capsule colonoscopy, and technologies for intraprocedural bowel cleansing. Many of these devices have the potential to reduce exam related patient discomfort, obviate the need for sedation, increase diagnostic yield, reduce learning curves, improve access to screening, and possibly avert the need for a bowel preparation.

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Key words: Endoscopy; Technology; Capsule colonoscopy; Colorectal cancer; Screening

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide with approximately 608 000 people dying each year[1]. In the United States alone there are approximately 1.14 million people alive who have a history of CRC and 1 in 20 will be diagnosed with cancer of the colon or rectum in their lifetime[2]. Unfortunately, this number is projected to increase by 62% by the year 2030[3].

Diagnosing CRC has been increasingly successful due to numerous advances in technology. One of the paramount technological advances has been the ability to directly visualize the gastrointestinal (GI) tract (and provide therapy) with the flexible endoscope. The earliest flexible endoscope, completely based on optical fibers, was

presented at the American Gastroscopy Society annual meeting in May 1957 by Hirschowitz[4]. This achievement was inspired by a paper published in 1954, entitled "a flexible fiberscope using static scanning", by Hopkins *et al*[5] at the Imperial College of Science and Technology in London. Building on this history of technological innovation, and driven by breakthroughs in electronics, material science, computational capabilities, sensing, and actuation strategies, many novel GI devices and diagnostic techniques have emerged. In this review, we will discuss a set of advanced endoscopic technologies for CRC screening that are either already available or close to clinical trial; and have the potential to reduce procedure related patient discomfort and increase diagnostic yield.

LIMITATION OF STANDARD GI ENOSCOPY

Flexible endoscopy is considered to be an effective method for early diagnosis and treatment of GI cancer, thus is a primary choice for screening programs. Few complications are associated with this technique, with cardiorespiratory problems related to sedation and analgesia being the most common (0.03%-20% incidence[6]). Less frequent complications include, infection (0.2% incidence[7]), bleeding (0.2%-2.1% incidence) or perforation (0.1% incidence[8]), which potentially require subsequent medications, transfusions, or endoscopic/surgical intervention to correct.

Based on the efficacy and low complication rate of flexible endoscopy, it is clear that the main clinical challenge facing GI endoscopy is one of distribution. Millions of people who may benefit from endoscopic CRC screening fail to have the procedure performed. The reasons cited include psychological barriers due to the indignity of the procedure, fear of procedure related pain, bowel preparation discomfort, and potential need for sedation[9].

How rational are these fears? From a mechanical perspective, the endoscope consists of a long and fairly stiff (compared to the compliant colon) tube with a steerable head. The colonoscope must easily navigate the colon curves and traverse the intestinal environment efficiently - meaning that a colonoscope must be simultaneously stiff and compliant. If the colonoscope is too stiff, it will deform the colon wall significantly at turns; yet, if it is too compliant there will be undesired buckling[10]. Since the colonoscope must be pushed from the back, while the tip is aimed along the lumen center, when the intestine bends, the shaft pushes against the colon wall until the lumen and its surroundings provide sufficient counter pressure to force the endoscope shaft to bend. This stretches the colon and often leads to "loop" formation, thus potentially causing substantial discomfort. In particular, looping occurs when the colonoscope continues to be advanced into the colon without a corresponding progression of the tip. This displaces the colon from its native configuration and stretches mesentery muscles. Looping of the endoscope has been shown to be responsible for 90% of the pain episodes in colonoscopy and increases the chance of tissue damage and perforation[11]. Special maneuvers can be performed to minimize this effect, making colonoscopy a procedure that requires a great degree of training, technical skill, and experience to safely perform[12,13]. Despite these techniques, even expert endoscopists can not always prevent all challenges or complications; partially because the flexible endoscope design is one of compromise and is not perfect for its intended purpose[10]. Additionally, the need for a bowel preparation acts as a potential deterrent due to the unpleasantness of ingesting powerful medications to clean the intestine. A detailed discussion on bowel preparation is beyond the scope of this review and will only be discussed briefly.

TOWARD MECHANISMS ENABLING PAINLESS COLONOSCOPY

Several colonoscope modifications have recently been presented with the common goal of preventing excessive force application to the colon wall and consequent looping. These devices can be categorized into two groups: those designed purely for visual inspection (visual inspection devices), and those that contain internal channels through which interventional devices (i.e., biopsy, snare, needle, *etc.*) can be passed (interventional devices). In addition to flexible colonoscope modifications, wireless capsule colonoscopy is emerging as a "patient-friendly" alternative technique for visual inspection of the colon. In this section we will provide an overview of recent advancements in these three device categories.

Visual-inspection-only advanced flexible colonoscopes

An example diagnostic-only device is the CathCam, whose development has been supported by Ethicon Endo Surgery, Inc., represented in Figure 1A. The CathCam is a nonsterile, disposable, multilumen catheter with a working length of 1.8 m and a diameter of 11 mm. Vision and illumination are provided by a 3-mm camera with six light-emitting diodes. The catheter is designed for single use, while the camera is reusable and is mounted into the catheter tip prior to the procedure. The channels of the CathCam accommodate the cables of the video camera and enable the system to provide suction, irrigation, and visualization. An accessory channel (2.8 mm in diameter) is provided for a looped guide wire (0.024-inch hinged lumen-seeking guide wire) that is advanced through the channel and into the lumen of the colon. The guide wire then serves to guide the catheter forward when the catheter is pushed[14]. This device has demonstrated 30%-40% peak force reduction in benchtop experiments and in live pigs, in comparison to standard colonoscopes[15] (Table 1).

Another advanced diagnostic flexible endoscope is the Aer-O-Scope™ (GI View Ltd., Ramat Gan, Israel), which is a pneumatic, skill-independent, self-propelling, and self-navigating disposable colonoscope[16], repre-

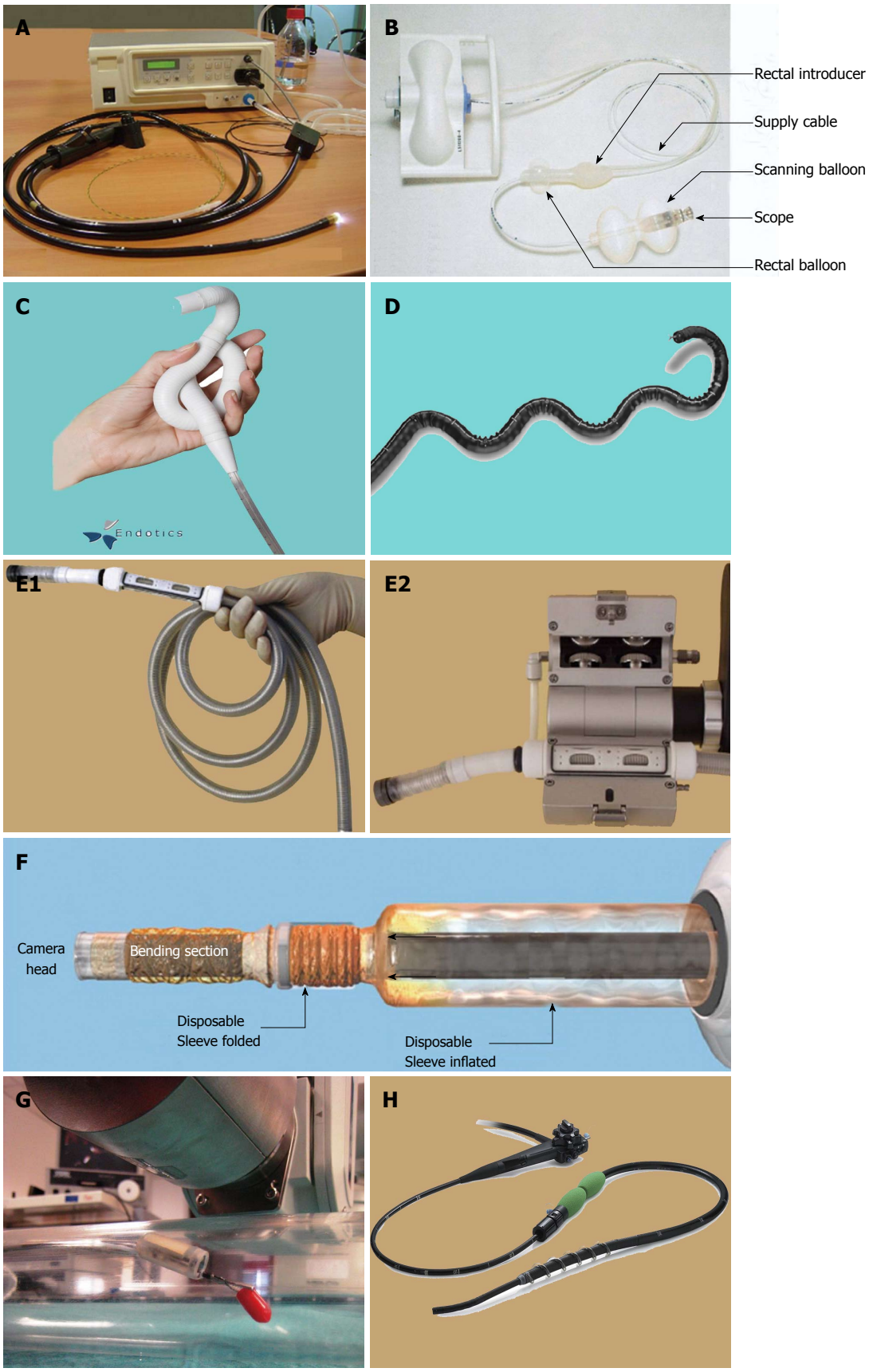

Figure 1 Figures of the devices. A: The CathCam system, reprinted from[15], with permission from Elsevier; B: The Aer-O-Scope™ system, reprinted from[18], with permission from Elsevier; C: The Endotics™ system, image courtesy of Era Endoscopy; D: The NeoGuide™ system, reprinted by permission from Macmillan Publisher Ltd.: The *American Journal of Gastroenterology*[11], copyright 2007; E: The Invendoscope™ system, reprinted from[24] with permission from Elsevier; F: The ColonoSight™ system, reprinted from[26] with permission from Elsevier; G: Magnetic endoscopic device described in[27]; H: The Endo-Ease™ overtube over a pediatric colonoscope, reprinted from[28], with permission from Elsevier.

Table 1 Main characteristics of advanced flexible colonoscopes

Technology	Advantages	Disadvantages	Human studies
CathCam	Disposable; peak force reduction with respect to standard colonoscope	No instrument channel; no steering	No
Aer-O-Scope™	Disposable; "tip pulling" locomotion; computer-aided control	No instrument channel; no steering	Yes[18]
Endotics™	Disposable; Steerable head; "tip pulling" locomotion; computer-aided control; thin tail	No instrument channel; average procedure time longer than colonoscopy	Yes[20,21]
NeoGuide™	Instrument channel; shape retention; 3D map of the device; computer-aided control	Reusable (need for cleaning); large diameter	Yes[11]
Invendoscope™	Disposable; instrument channel; "tip pulling" locomotion; computer-aided control	Diameter similar to a colonoscope	Yes[24,25]
ColonoSight™	Disposable; instrument channel; "tip pulling" locomotion	Diameter similar to a colonoscope	Yes[26]
Magnetic endoscopic device	Instrument channel; "tip pulling" locomotion; computer-aided control; thin tail	Complexities related to magnetic control	No
Endo-Ease™	Facilitates cecal intubation	Large diameter	Yes[29]
ScopeGuide™	3D real-time visualization of the device	Standard colonoscope	Yes[32,33]

sented in Figure 1B. The device is composed of a rectal introducer, a supply cable, and an endoscope embedded within a scanning balloon that serves as its vehicle. The rectal introducer is a hollow silicon tube (1.7 m in length, 19 mm in diameter) with a silicone balloon (80 mm in diameter) attached to its outer surface. The introducer is inserted into the rectum with its outer balloon, and the endoscope and its vehicle balloon are passed through the hollow tube of the introducer. The silicone balloon on the introducer seals the anus to prevent gas leakage. CO_2 is insufflated between the two inflated balloons, and gas pressure advances the vehicle balloon, endoscope, and a trailing supply cable. The supply cable on the endoscope/scanning balloon is a flexible polyurethane multi-lumen catheter, 5.5 mm in diameter, coated with a hydrophilic material, which supplies the electro-optical capsule (the scope) and its vehicle balloon with electricity, air, water, and suction. The volume of the vehicle/scanning balloon is pressure-regulated. The balloon is designed to adapt to the shape of the colon as it travels forward. The balloon has a large predetermined maximal volume and thin wall (8 μm) that help it adapt to the shape of the colon in response to pressure exerted by the colonic walls. These features allow the balloon to maintain the seal with the colon wall, so that gas introduced on one side of the balloon does not escape to the other side. This allows a pressure gradient to develop from behind the balloon to in front of it during forward propulsion, and vice versa during withdrawal. Since the tip has no steering capabilities, an omnidirectional colonoscopic viewing system has been installed on the tip of the latest prototype[17]. The Aer-O-Scope™ is intended to be used for diagnostic purposes and therefore does not have a working channel for therapeutic instruments. In a preliminary pilot feasibility study[18], the Aer-O-Scope™ effectively intubated the cecum in 10 out of 12 subjects (83%) with an average time to cecum of 14 min ± 7 min.

Another approach, inspired by Geometer moths, is inchworm locomotion[19]. Inchworm locomotion is similar to the motion utilized in double balloon endoscopy where there is lengthening and shortening along two anchor points (distal anchor and proximal anchor). The Endotics™ system (Era Endoscopy s.r.l., Pisa, Italy) effectively utilized inchworm locomotion and consists of a disposable robotic probe as a head, a steerable tip, a flexible body (17 mm in diameter), a thin tail (7.5 mm in diameter), and a control box with an electro-pneumatic connector. The Endotics™ endoscopic device is represented in Figure 1C. The head hosts both a vision system, including camera, light emitting diode light source and channels for a water jet and air insufflation/suction. The operator can steer the head of the robotic colonoscope 180° in every direction, elongate the body of the probe in order to move it forward along the intestine, and can control rinsing, insufflation, and suction. A semiautomatic sequence of actions is implemented to move the probe like an inchworm, wherein two vacuum anchors located in the proximal and distal ends of the probe are sequentially actuated between extensions and retractions of the central body. Experiments in a phantom model with embedded force sensors demonstrated 90% lower force application than conventional colonoscopy[20]. A human study[21] with 71 unsedated patients demonstrated that this system has a comparable diagnostic accuracy to colonoscopy, and does not require sedation. The frequency of successful procedures (i.e., reaching the cecum), the total procedure time, and the need for sedation were recorded in this study. In 13 cases (18%), the device was unable to reach the cecum and the average procedure time was 45.1 ± 18.5 min for the Endotics™ system compared to 23.7 ± 7.2 min for traditional colonoscopy ($P < 0.0001$).

Currently, the lack of tissue interaction makes it improbable that diagnostic-only devices will completely replace traditional colonoscopes. Only prospective comparative outcome trials will be able to conclusively determine whether a diagnostic-only device (followed by conventional colonoscopy for potential therapeutic intervention) will be preferable to conventional colonoscopy with both diagnostic and therapeutic capabilities.

Interventional colonoscopes with alternative propulsion mechanisms

A first subset of this class of colonoscopes consists of shape retention devices, which are essentially tubes that

are initially flexible and can be stiffened when desired. While several examples of devices utilizing this principle are described in literature[10], only one has reached the clinical trial stage. This is the NeoGuide™ from NeoGuide Systems Inc. (a company based in San Jose, California, United States that was acquired in 2009 by Intuitive Surgical of Sunnyvale, CA, United States). The NeoGuide™, represented in Figure 1D, consists of a 173 cm-long endoscope composed of sixteen 8 cm-long independent vertebrae. Each segment can be directed to assume a right, left, up, down, circular curve, or a combination of these motions. During manual insertion of the device, the position and angle of the scope tip are encoded into a computer algorithm. As the colonoscope is advanced, the computer directs each successive vertebra to take the same shape that the tip had at a given insertion depth. The insertion tube thus changes its shape at different insertion depths in a "follow-the-leader" manner. By controlling the shape of the insertion tube, the NeoGuide™ does not rely on conventional pushing against the colon wall to maneuver. The cross-sectional diameter of the NeoGuide™ insertion tube is approximately 14 mm at the tip, increasing to approximately 20 mm at the proximal shaft of the scope (the working channel is 3.2 mm). The mechanical valves that control insufflation, suction, or water irrigation are the same as in conventional endoscopes. Biopsies and therapeutic maneuvers are conducted with the scope in passive mode; where the shape and stiffness of the endoscope is the same as that of a standard colonoscope. *In vitro* evaluation of the NeoGuide™ system[22] showed significantly less looping and lateral force required for advancement than procedures with a standard colonoscope. An initial clinical trial of 10 sedated patients demonstrated a looping rate of 40%. Although looping was defined as extensive in three of four cases, it was successfully straightened under computerized 3D imaging and the cecum was reached in all patients[11]. The 3D map images generated by the NeoGuide™ endoscopy system provide information regarding tip position, insertion tube position, and colonic looping[23]. These additional pieces of information may contribute to a safer and more comfortable procedure for the patient.

Another computer-aided colonoscope is the disposable Invendoscope™ (Invendo Medical GmbH, Kissing, Germany). The colonoscope, represented in Figure 1E, has a working length of 210 cm and the internal functional endoscope is covered by several layers, starting with a 10-mm diameter sheath. The sheath is covered by double layers of an "inverted sleeve" that provides the propulsion mechanism. Eight drive wheels in the driving unit grip the inner layer of the inverted sleeve and rotate, causing it to move forward. The "inverted sleeve" mechanism causes the colonoscope to "grow" at a position 10 cm below the distal end. Similarly, when the colonoscope is being retracted, the drive wheels rotate in the opposite direction and the endoscope "shrinks". This technology, combined with a small bending radius, was designed to reduce the forces exerted on the walls of the colon, with the goal of minimizing patient discomfort - even without sedation. Other than these mechanisms, the Invendoscope™ functions in a manner similar to conventional endoscopes, allowing for insufflation, rinsing, and suction. It also has a 3.2-mm working channel, allowing for therapeutic procedures to be performed.

A pilot study of 34 healthy volunteers (19 men; mean age 49.7 years) demonstrated that the Invendoscope was able to reach the cecum without any sedation in 80%-90% of cases[24]. This result was supported by a prospective single-arm study of 61 healthy volunteers (34 men and 27 women; mean age 57.5 years) undergoing screening colonoscopy, with a cecal intubation rate of 98.4% with less than 5% of patients requiring sedation[25].

The ColonoSight™, a disposable, self-propelling device based on IntraPull and ProtectiScope technologies by Stryker, Inc., has three working channels: a 3.7-mm-wide channel for suction and insertion of accessory tools, a channel for irrigation, and a channel for insufflation (Figure 1F). A disposable sleeve anchored at the proximal end of the device envelopes the endoscope, protecting it from contamination. The IntraPull mechanism generates a force close to the tip of the scope by pumping compressed air inside the sleeve controlled by depressing a foot pedal. The material of the sleeve does not allow expansion, and therefore the increased pressure inside the sleeve creates a force directed toward the tip of the colonoscope, thus pushing the tip forward as the folded part of the sleeve is deployed. The maximum force generated is 4.9 N (0.5 kgf) whereby the maximum force generated with a standard colonoscope is approximately 44 N (4.5 kgf). Once the endoscopist releases the foot pedal, the sleeve is deflated and the applied force is removed. A pilot prospective study of 178 participants (48 women and 130 men; age 51.8 ± 10.7 years) reported a success rate of 90% in reaching the cecum without complication of bleeding or perforation. All patients in the study were sedated using midazolam, mepiridine, or propofol; therefore, no data on patient discomfort was reported[26].

With the exception of the Endotics™ system, tip deflection of the above devices are accomplished by wires travelling the length of the device - effectively creating a minimum outer endoscope diameter (lower boundary) of approximately 10 mm. If however, one could "pull" rather than "push" the endoscope, the outer diameter lower boundary may be reduced further; limited by the space needed for the therapeutic channel and the electrical connections to the vision module[21]. Magnetic steering and control was applied to an endoscopic device containing a tip-mounted magnetic camera (diameter 11 mm, length 26 mm) connected to an external control box by a 5.4-mm diameter multi-lumen soft tether[27]. This connection was used for insufflation, passage of a therapeutic instrument, activation of a lens cleaning mechanism, and for operating the vision module. The external magnet was held by a 7 degree-of-freedom (DoF) robotic arm that was controlled in real-time by the endoscopist. The external magnet driving the endoscopic device is represented in Figure 1G.

A magnetic field sensor was also embedded in the device head to enable localization and closed loop control. The magnet capsule system allowed for "pulling" of the device throughout the colon thus eliminating the need for structural cables traveling the length of the device and the need for pushing the endoscope at the base in order to forward advance the scope. This approach would appear to prevent looping and reduce stretching of the colon wall. These advantageous characteristics are enhanced by dramatic reduction in both the bending stiffness of the shaft and in the mass of the proposed device (from 1240 g for a standard colonoscope to 34 g, including the soft tether), while retaining the therapeutic capabilities provided by a conventional colonoscope. The device has been tested in *ex-vivo* colon models and animal trials with encouraging results. Human trials are planned, but have not yet been conducted.

Another noteworthy mechanism for locomotion, designed to improve cecal intubation rate when standard colonoscopy fails, is the Spirus Endo-Ease™ system[28]. The Endo-Ease™ system, represented in Figure 1H, consists of a 90 cm disposable flexible plastic overtube with a 5-mm soft spiral thread at its tip. The overtube is designed with a 13-mm inner diameter to host a variable-stiffness pediatric colonoscope. Clockwise rotation of the overtube, which mimics the motion of a corkscrew, pleats the bowel onto the external surface of the tube. Bowel pleating is accomplished without apparent twisting of the lumen because the mesentery attachment resists the rotation. In a preliminarily clinical trial[29] of 22 patients (the median age 68 years, 58% men) with incomplete colonoscopy secondary to redundancy of their colon, the cecal intubation rate was reported in 22 patients (92%) with a median time to cecum of 14.2 min (range 6-30 min). No complications were reported.

The same principle was used in a computer-aided colonoscope[30] where two sections with a spiral thread rotate to achieve forward motion. While preliminary qualitative data about *in vivo* experience shows the feasibility of this approach (forward speed of 11 mm/min), clinical trials have not been reported to date.

A common theme in reducing discomfort associated with standard colonoscopy appears to be shifting the location of the propulsive force from the base of the device outside of the patient (i.e., "pushing", typical force profile available in literature[31]) to the tip of the colonoscope inside the patient (i.e., "pulling"). The latter can result in improved alignment of the direction of the applied force with the desired direction of forward tip motion. Several devices have demonstrated that this technique can reduce forces applied to the colon wall during insertion, which is believed to correlate with a reduction in patient discomfort and the risk of colon perforation. Diverse methods for reducing tip forces have been proposed, ranging from pneumatic pressure[22,25,26] to robotic locomotion[21] to magnetic fields[27]. While it remains to be seen which of these technologies will achieve market penetration and widespread clinical use; the thin tether of the last two devices described previously appears advantageous.

An alternative approach to minimize looping of the endoscope may lie in the endoscopists ability to "visualize" the shape of the endoscope as she or he advances it throughout the colon. A commercially available endoscopic system from Olympus, the ScopeGuide™ (CF-H180DL), integrates electromagnetic tracking into a standard colonoscope and provides a real-time rendering of the endoscope shape in free space on a display. A recent study[32] comparing this platform with standard colonoscopy reported reduced cecal intubation time (181 s *vs* 216 s; $P < 0.05$) and reduced patient discomfort on a 10-point visual analog scale (2.44 *vs* 1.85; $P < 0.05$). Unfortunately, other studies[33] of expert endoscopists utilizing this device have not reproduced these results; leading one to believe that the application of the system may be limited to novices.

In regard to reducing the learning curve, computer-aided devices or image guided techniques[18,21,23,25,27,32] have the potential to make colonoscopy less technically challenging for novices. Haptics has also been explored with promising results to facilitate colonoscopy[34]. In particular, lumen detection by real-time image analysis was used to provide a feedback force at the user interface that should aid the physician to steer the endoscope tip toward the lumen. Given the increasing role of technology in healthcare, one day it may be possible for examinations to be carried out remotely (i.e., patient and provider are not in the same physical location) by health care professionals in the community in order to meet the increased demand for screening colonoscopy.

Wireless capsule colonoscopy

Trends in consumer electronics such as miniaturization, low power operation, and wireless technologies have paved the way to the introduction of wireless capsule endoscopy (WCE) in 2000[35]. This technology has now become essential for small bowel inspection[36] and it is trying to expand its reach for use in the large intestine with purposely developed capsule devices, such as the PillCam™ Colon by Given Imaging (Yokneam, Israel). The effectiveness of the second generation of PillCam™ Colon[37] as an alternative to colonoscopy for CRC screening has been analyzed in a recent editorial[38] that appeared in this journal on the basis of recent clinical trials. Although the sensitivity for polyp detection for the PillCam™ Colon is close to 90%, specificity remains disappointingly low due to a substantial rate of false-positive results[39]. Therefore, despite clear patient comfort benefits provided by WCE, colonoscopy is still the gold-standard for CRC screening[40].

A main research stream for WCE toward the final goal of replacing flexible endoscopy consists in providing the capsule with the possibility to be remotely controlled and manipulated. Magnetic coupling is one of the few physical phenomena capable of transmitting actuation forces across a physical barrier. Remote magnetic ma-

nipulation has been adopted to steer capsule endoscopes by several research groups worldwide. Given imaging has investigated the use of a handheld external magnet to translate and orient a capsule in the upper GI tract using a modified version of PillCam™ Colon, which was half-filled with magnets[41]. This demonstrated the feasibility of magnetic steering, but revealed that more research was required to increase the reliability and accuracy of magnetic control[42]. An alternative technique for generating the external magnetic field, jointly developed by Olympus and Siemens, involved use of a magnetic resonance imaging scanner to create the field and field gradients[43]. In a recent nonrandomized blinded pilot study comparing a capsule device to traditional endoscopy for evaluation of the stomach, the overall diagnostic yield was similar for both methods[44].

A robotic navigation system - commonly used for cardiovascular clinical procedures (Niobe™, Stereotaxis, Inc, United States) - was used to control the orientation of a wireless capsule endoscope throughout the entire GI tract in several *ex-vivo* and *in-vivo* animal trials[45-47]. This technique was validated by equipping a PillCam™ with a custom-made coaxial magnetic shell[48] glued to its external surface. *In vivo* tests performed with 3D fluoroscopic localization demonstrated an accuracy of 1° in orientation[47]; however, the Niobe™ does not allow for field-gradient control, thus controlled translations of the capsule are not possible - this is an important limitation for this approach.

Magnetic control over position and orientation of a capsule inside the colon was demonstrated by coupling permanent magnets - one embedded inside the endoscopic capsule and the other connected as the end effector of a 6 DoF industrial robotic arm in an animal pilot study[49]. The same kind of magnetic coupling was used by the first ever reported wireless therapeutic endoscopic capsule[50]. This pilot study in a porcine model demonstrated the feasibility of wireless controlled deployment of a surgical clip to close an iatrogenic bleed in the colon. In a different study[51], the combined use of external static magnetic fields and internal actuation to move small embedded permanent magnets allowed for wirelessly controlled precise camera steering. This approach was used to obtain a full 360° view in the gastric cavity and a 45° span inside the colon.

Overall, this body of literature indicates that precise capsule manipulation can be achieved by means of magnetic coupling and that, if relevant clinical evidence will further support this approach, the next generation of wireless capsule endoscopes may eventually have a concrete potential to replace traditional colonoscopy, at least for CRC screening.

TOWARD MECHANISMS ENABLING UNPREPARED COLONOSCOPY

A significant common disadvantage of both conventional and novel colonoscopes is the need for bowel preparation. An innovative device addressing this challenge is the ClearPath™ system[52] that consists of a control cabinet and a disposable unit. The control cabinet includes a peristaltic pump, a controller, and a pinch valve that enables control of suction. The disposable element consists of a multi-lumen, custom-made, extruded tube. The tube has two channels: one that supplies water for irrigation and one that provides suction. When attached to the standard colonoscope, the ClearPath™ system adds an additional 6 mm to the outer diameter of the standard colonoscope. Water for irrigation flows through four 0.6 mm nozzles in the distal head and debris is evacuated through a single 18 mm^2 cross-sectional aperture. Preliminary animal trials on partially prepared domestic swine demonstrated effective intraprocedural colon cleaning with no immediate mucosal damage, acute complication (i.e., perforation), or delayed adverse consequence.

An alternative approach[53] for cleaning the colon during colonoscopy consists of a disposable soft-tipped catheter with a water jet spray that can be advanced, under direct vision, through the accessory channel of the standard colonoscope and into fecal matter. When the water jet is activated the solid stool can be broken apart for removal by suction. The water jet catheter tip has 4 radial nozzles through which the water is pumped. Feasibility studies in the unprepared colons of anesthetized pigs demonstrated effectiveness; however, mucosal trauma, bleeding, perforation, clogging of the colonoscope suction channel, and electrolyte imbalances may limit the overall impact.

A similar approach for intraprocedural bowel cleansing[54], still based on a disposable catheter, has been developed by Medjet Ltd., Tel Aviv, Israel. The MedJet digestive tract lumen cleaning device provides controlled delivery of a supersonic two-phase jet of micro-droplets consisting of minimal amounts of saline solution and CO_2. The solution is accelerated through the catheter, and enhances visibility by clearing away stool, secretions, or blood; and by disintegrating small particles for suctioning through the working channel of the endoscope during colonoscopy. In a recent clinical study[54], the MedJet was reported to be effective - offering significant improvement in bowel cleansing in 32 patients with no device-related adverse events.

In summary, there have been promising advances in the development of devices utilized for CRC screening with the devices designed to achieve a common goal to improve the way we directly view the colon (and possible intervene on any pathology present). The main principle that must be kept in mind with any new device is patient safety. Secondary principles that continue to be addressed include ease of use, reduction of the learning curve, improvement in colonic visualization, improvement in patient procedural comfort, better access to endoscopic screening, and possibly obviation of the need for a bowel preparation. With technology improving at a rapid pace, it may not be long before a disruptive innovation takes hold and we see the conventional colonoscope as an item on the shelf in a medical museum.

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FIELD OF VISION

MicroRNA-feedback loop as a key modulator of liver tumorigenesis and inflammation

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Abstract

A recent work of Iliopoulos *et al* published in *Cell* highlighted a circuit orchestrated by microRNAs (miRNAs) that results in liver tumorigenesis and inflammation. This feedback loop, governed by miR-24 and miR-629, promotes a hepatocyte nuclear factor-4α transient inhibition resulting in miR-124 induction and signal transducer and activator of transcription 3 activation. These promising data support the use of miRNA mimics or inhibitors as potent therapeutic approaches in liver cancer.

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Key words: Hepatocyte nuclear factor-4α; MicroRNA; Interleukin-6; Signal transducer and activator of transcription 3; Hepatocellular oncogenesis

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COMMENTARY ON HOT TOPICS

Hepatocyte nuclear factor-4α (HNF-4α) belongs to the nuclear receptor superfamily of ligand dependent transcription factors NR2A1[1]. It is expressed in the kidney, intestine and pancreas. HNF-4α is most highly expressed in the liver[2-5], where it binds to the promoter of 12% of genes expressed in the adult liver[6]. It plays a key role in liver development during gastrulation[7] and orchestrates the parenchyma formation in the adult liver[8]. It also guides hepatoblasts to hepatocyte differentiation[9]. The conditional disruption of HNF-4α in mouse liver showed that HNF-4α regulates a number of genes involved in lipid, glucose, amino acid and xenobiotic metabolisms[4]. Related to its central role in liver physiology, HNF-4α has been reported to be involved in liver oncogenesis; however, its role is still controversial. A study by Xu *et al*[10] suggested that HNF-4α expression was increased in human hepatocarcinoma (HCC), which contrasted with other studies that reported that the HNF-4α level was decreased in HCC[11-13]. HNF-4α has also been associated with cancer and inflammation in the colon: its intestinal disruption in mouse revealed that HNF-4α protects against colitis and inflammatory disease[14,15] and promotes tumorigenesis[15]. Liver cancer is a clear example of an inflammation-related cancer[16-19]; therefore, it does not seem surprising that HNF-4α could also orchestrate an inflammatory program in the liver, as was shown last year by Wang *et al*[20] in experiments performed on hepatocarcinoma cells exposed to interleukin (IL)-6.

In a recent paper published in *Cell*, D. Iliopoulos' Laboratory aimed to decipher the epigenetic circuit centered on HNF-4α, which promotes liver oncogenesis[21]. They particularly focused on microRNAs (miRNAs), which are small RNAs produced from non-coding DNA regions that control gene expression by binding to the 3' untranslated region (3' UTR) of target messenger RNAs (mRNAs). miRNA binding results in the degradation of the mRNA and/or the repression of its translation[22].

Since their discovery in 1994 in *Caenorhabditis elegans*[23], miRNAs have become an expanding area of research, and evidence supports the view that miRNAs are key regulators of many physiological processes, e.g., growth, proliferation and differentiation[24-27]. They are also implicated in the initiation and progression of various cancers[28-30]. Concerning HCC, microtranscriptomic analyses revealed a miRNA signature of liver cancer, i.e., miR-122 loss[31] or miR-221 upregulation[32], and a number of studies support the view that miRNAs direct HCC progression through the induction of cell proliferation or metastasis and the suppression of apoptosis[33]. Taken together, these data highlight miRNAs as promising diagnostic and therapeutic targets for HCC.

The work of Maria Hatziapostolou is a robust description of the molecular factors underlying HNF-4α loss-mediated hepatocarcinoma based on *in vitro* and *in vivo* experiments and confirmed on HCC human samples. Prior to the precise deciphering of hepatocarcinoma molecular origins, the authors confirmed that the silencing of HNF-4α was sufficient to increase cell colony formation and invasion of nontransformed immortalized human hepatocytes (IMH) and human liver cancer cell lines. On their own, the injection of IMH cells with a preliminary transient inhibition of HNF-4α in immunosuppressed mice resulted in tumor apparition. Interestingly, HNF-4α was maintained at a low level 55 d after cell xenografts, suggesting that transient silencing of HNF-4α initiated a feedback loop that stably maintained cell transformation and suppressed its own expression.

What are the factors involved in HNF-4α silencing? An miRNA screening was performed on a luciferase reporter gene containing the 3' UTR region of HNF-4α, which revealed that miR-24 and, to a lesser extent, miR-629, were HNF-4α repressors. The transient expression of both miRNAs, the equivalent of HNF-4α silencing, transformed IMH cells, increased their invasiveness *in vitro* and promoted the appearance of tumors in mice. Strikingly, both miRNAs were induced following HNF-4α silencing, supporting the involvement of these miRNAs in the HNF-4α- dependent feedback loop.

Interestingly, miR-24 and miR-629 possess a binding motif for signal transducer and activator of transcription 3 (STAT3) in their promoters, a well-known effector of IL-6-dependent cancer inflammation[34] (for a review), particularly in liver cancer[35] (for a review). In brief, the binding of IL-6 to its receptor, IL-6R, activates the Janus kinase family, which phosphorylates STAT3 on tyrosine residues. STAT3 could then form homodimers before translocation into the nucleus to promote the expression of an inflammatory program[36] (for a review). Here, the authors showed that in response to IL-6, STAT3 binding to miR-24 and miR-629 promoters increased, resulting in miRNA expression. In turn, miR-24 expression and HNF-4α silencing enhanced the phosphorylation status of STAT3, and thus its activation. STAT3 and IL-6 are two other members of the HNF-4α feedback circuit promoting liver oncogenesis; an antibody against IL-6 blocked all the HNF-4α silencing effects that they observed.

The next step consisted in screening of miRNAs possessing an HNF-4α binding site, which identified miR-124 as a preferential HNF-4α target. miR-124 was, in fact, an integral part of the feedback loop, because its inhibition by an antisense as-miR-124, or following HNF-4α silencing, enhanced STAT3 phosphorylation coupled to an induction of IL-6 secretion and expression of IL-6R, a direct target of miR-124.

The relevance of the loop miR-24/miR-629/HNF-4α/miR-124/STAT3 was confirmed *in vivo* with a mouse model of HCC induced by diethylnitrosamine. During tumor progression, the HNF-4α level was initially dropped off after four weeks, followed by miR-124 repression and inversely by miR-24 and IL-6R induction, which supports the essential role of the HNF-4α loop in tumor progression. One of the important finding of this study was that an injection of miR-124-mimic encapsulated into liposomes in this model was sufficient to limit tumor growth, even if this mimic was administered four weeks before analysis. miR-124 mimics were also able to prevent tumor development. Taken together, these results argue in favor of miR-124 delivery as a potent strategy for treating or preventing HCC. Although, to date, the efficacy of miRNA mimics have not been demonstrated *in vivo*, this approach could be attractive. The prerequisite for this approach is that the complementary passenger RNA in the double stranded miRNA mimic does not create a new miRNA that could induce off-target effects[37,38].

The second exciting idea from this study relies upon the need for an inflammatory context to optimally disrupt the HNF-4α epigenetic circuit and promote tumorigenesis. If the tumors developed in a mouse model lacked STAT-3 in the liver, the tumors were smaller when the HNF-4α loop was less inhibited. This supports the hypothesis that HNF-4α loss engages an anti-tumorigenic program and a STAT3-dependent anti-inflammatory program, both orchestrated by the trio miR-24/miR-629/miR-124, to induce hepatocyte transformation. Such an epigenetic loop has already been described by the same authors for breast cancers, in which cell transformation was dictated by an epigenetic switch achieved by IL-6, nuclear factor-κB and the miRNA let-7[39].

Finally, the authors demonstrated the relevance of their model for human liver oncogenesis: more than 50% of HCC samples from patients showed a loss of HNF-4α and of miR-124, accompanied by an increase in miR-24 and IL-6R expression; all these parameters were associated with a higher level of phospho-STAT3 status in relation with IL-6 and IL-6R expression. As a confirmation of the HNF-4α circuit's key role in HCC progression, the authors showed that the activity of the HNF-4α circuit becomes more deregulated as the HCC grade increased.

In summary, this work strongly argues for a tumor suppressor role of HNF-4α in liver, as was already sh-

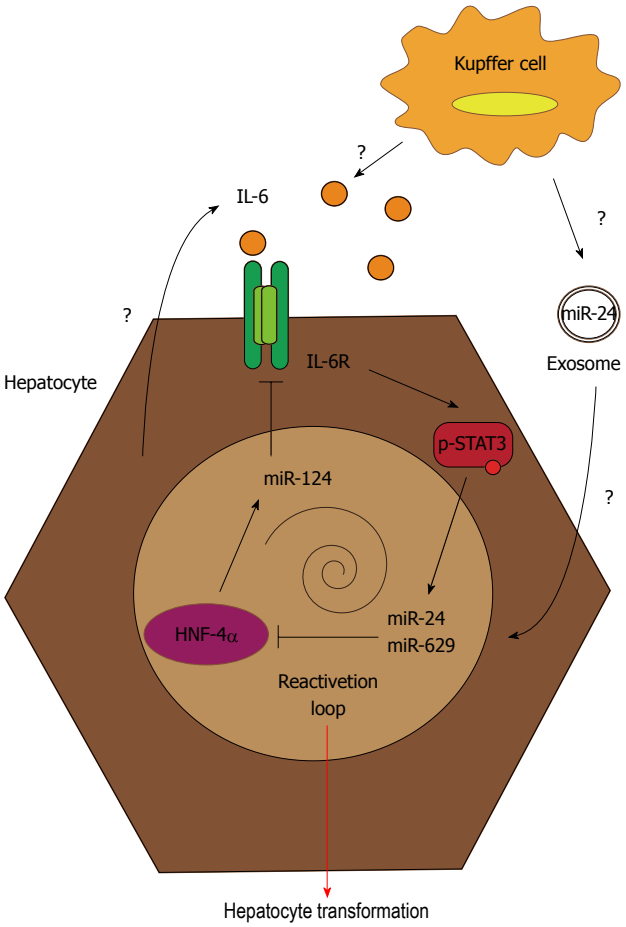

Figure 1 From transient hepatocyte nuclear factor-4α inhibition to stable hepatocyte transformation. The transient inhibition of hepatocyte nuclear factor-4α (HNF-4α) following miR-24 and miR-629 induction promotes stable repression of the pro-tumorigenic HNF-4α feedback circuit. This results in miR-124 inhibition, followed by an increase in interleukin (IL)-6 secretion and IL-6R expression. These two events lead to hyperphosphorylation of signal transducer and activator of transcription 3 (STAT3), which auto-amplifies the process through the re-induction of miR-24 and miR-629.

own[12], and supports the key role of this transcription factor in limiting the tumor inflammatory environment by inhibiting the IL-6/STAT3 pathway. The authors described the interconnection between the trio miR-24/miR-629/miR-124 and HNF-4α as a key regulator of HCC progression (Figure 1). In 2010, Takagi *et al*[40] described a crosstalk between HNF-4α and miR-24 in a liver metabolic loop for the control of bile acid synthesis. In brief, bile acids induce reactive oxygen species generation, resulting in the activation of the mitogen-activated protein (MAP) kinase pathway. In turn, miR-24, induced by the MAP kinases, downregulates HNF-4α, and, thus, the expression of bile acid-synthesizing enzymes. Hepatitis B virus (HBV) and alcohol abuse, two etiological agents associated with HCC development, contribute to oxidative stress in the liver. In consequence, miR-24 inhibition and, thus, reactivation of the HNF-4α feedback circuit in HCC could also be performed using antioxidants, a class of therapeutic agents currently used for HBV patients[41].

The take-home message of this study is that a transient signal at any level of the feedback circuit (HNF-4α or miR-124 silencing, miR-24 overexpression or IL-6 secretion) stably induces hepatocyte transformation, which could be auto-amplified by this feedback loop, and lead to chronic inflammation, predisposing a patient to liver cancer development and immune escape[19]. The activation of the IL-6/STAT3 axis is, at least in part, caused by a hepatocyte source, as shown by the *in vitro* experiments, but this could also be boosted by IL-6 secretion by non-parenchymal liver cells like Kupffer cells. Another possible scenario for the disruption of HNF-4α circuit by tumor environment could be the secretion of exosomes containing miRNA, in particular miR-24, by immune cells. Exosomes are small extracellular vesicles derived from the multivesicular body-sorting pathway, which are produced by various cells like epithelial cells, immune cells and tumor cells. Recently, it has been shown that some miRNAs, particularly those deregulated in cancer, are encapsulated into exosomes and play a role as cell-cell mediators[41]. In particular, miR-24 has been detected in exosomal structures, and a similar mechanism might exist in liver, permitting miR-24 to be delivered to hepatocytes by neighboring cells.

The most promising result of this study was that intravenous delivery of miR-124 mimic encapsulated into liposomes was able to prevent and reduce the growth of liver cancer. To date, few works have demonstrated the potency of an miRNA-based anticancer approach. However, the great advantage of HCC is that mimics or inhibitors against miRNAs are preferentially distributed to the liver[42]. For that reason, the first miRNA-based therapy authorized in clinical trials is the anti-miR-122 compound Miravirsen, indicated for the treatment of hepatitis C virus (HCV), which uses miR-122 in hepatocytes for its replication. This clinical trial follows promising results obtained in primates infected with HCV and treated with an anti-miR-122[43], which was well-tolerated[44]. Concerning the HNF-4α circuit, the authors showed the efficiency of a mimic of miR-124 to perturb HCC development, but we could also conceive the injection of inhibitors against miR-24 and miR-629 to avoid HNF-4α loss. Even if miRNA inhibitor delivery appeared as a promising therapeutic approach, the use of these modulators poses a number of challenges. The question of treatment specificity could be raised. In fact, an miRNA could target multiple mRNAs[45,46], and, thus, many cellular pathways, pro- or anti-tumorigenic. It could be difficult to restrict the effect of one miRNA to only one target of interest. However, this notion could be generalized to many other treatments currently used, such as proteasome[47] or histone deacetylase inhibitors[48], which have demonstrated efficacy. Another major disadvantage could be the inhibition of a non-identified target. Their delivery could also be an important challenge, although many improvements have appeared recently, with chemical modifications limiting their degradation by nucleases[49-51]. The other problem is that these inhibitors preferentially target the liver and other tissues are more difficult to enter[42]. Finally, these inhibitors could also

be toxic[52,53]. As suggested by the data of Hatziapostolou, mimics or inhibitors could be delivered in a free formulation, or protected into liposomes or even into exosomes, as was shown for siRNAs[54]. In conclusion, miR-124-based treatment could be a therapeutic strategy of choice for HCC, as an alternative to the anti-STAT3 approach[55]. This therapeutic option has the great advantage of targeting the inflammatory, as well as the metabolic initiators, of HCC, and its efficiency demonstrated in this work argue for an epigenetic switch as the major event underlying cancer initiation.

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TOPIC HIGHLIGHT

Anca Trifan, Professor, *Series Editor*

Update on adrenal insufficiency in patients with liver cirrhosis

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Abstract

Liver cirrhosis is a major cause of mortality worldwide, often with severe sepsis as the terminal event. Over the last two decades, several studies have reported that in septic patients the adrenal glands respond inappropriately to stimulation, and that the treatment with corticosteroids decreases mortality in such patients. Both cirrhosis and septic shock share many hemodynamic abnormalities such as hyperdynamic circulatory failure, decreased peripheral vascular resistance, increased cardiac output, hypo-responsiveness to vasopressors, increased levels of proinflammatory cytokines [interleukine(IL)-1, IL-6, tumor necrosis factor-alpha] and it has, consequently, been reported that adrenal insufficiency (AI) is common in critically ill cirrhotic patients. AI may also be present in patients with stable cirrhosis without sepsis and in those undergoing liver transplantation. The term hepato-adrenal syndrome defines AI in patients with advanced liver disease with sepsis and/or other complications, and it suggests that it could be a feature of liver disease *per se*, with a different pathogenesis from that of septic shock. Relative AI is the term given to inadequate cortisol response to stress. More recently, another term is used, namely "critical illness related corticosteroid insufficiency" to define "an inadequate cellular corticosteroid activity for the severity of the patient's illness". The mechanisms of AI in liver cirrhosis are not completely understood, although decreased levels of high-density lipoprotein cholesterol and high levels of proinflammatory cytokines and circulatory endotoxin have been suggested. The prevalence of AI in cirrhotic patients varies widely according to the stage of the liver disease (compensated or decompensated, with or without sepsis), the diagnostic criteria defining AI and the methodology used. The effects of corticosteroid therapy on cirrhotic patients with septic shock and AI are controversial. This review aims to summarize the existing published information regarding AI in patients with liver cirrhosis.

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Key words: Liver cirrhosis; Adrenal insufficiency; Septic shock; Corticosteroid therapy

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INTRODUCTION

Adrenocortical dysfunction in patients with liver cirrhosis has been described for over half a century[1], but was ignored until a decade ago when several studies reported that some septic patients had an inappropriately low response of adrenal glands to stimulation, and treatment with corticosteroids decreased mortality[2,3]. Relative adrenal insufficiency (RAI) is the term given to inadequate production of cortisol with respect to the severity of

illness[4,5]. More recently, another term, namely critical illness related corticosteroid insufficiency (CIRCI) defined as "inadequate cellular corticosteroid activity for the severity of the patient's illness"[6], has been used. Despite a large number of published studies during recent years, the concepts of RAI and CIRCI are still under debate.

Liver cirrhosis is a major cause of mortality worldwide[7], often with septic shock as the terminal event[8]. It is a well-established fact that cirrhotic patients have increased susceptibility to bacterial infections[9]. Both cirrhosis and septic shock share many hemodynamic abnormalities such as hyperdynamic circulatory failure, decreased peripheral vascular resistance, decreased mean arterial pressure, increased cardiac output, hyporesponsiveness to vasopressors, increased levels of proinflammatory cytokines [interleukine (IL)-1, IL-6, tumor necrosis factor-α (TNF-α)][5,10,11] and, consequently, several studies reported that adrenal insufficiency (AI) is common in critically ill cirrhotic patients[8,12-14]. Furthermore, AI may occur in compensated and decompensated cirrhosis without sepsis[14-20] or in early and late post-liver transplantation (LT)[12,21-23]. Nowadays, liver cirrhosis is considered to be among the major groups of high-risk diseases with a predisposition to AI[24]. The term hepatoadrenal syndrome is used to define AI in patients with advanced liver disease with sepsis and/or other complications[12,15], suggesting that adrenocortical insufficiency may be a feature of liver disease *per se*, with a different pathogenesis from that occurring in septic shock.

Mechanisms of AI in cirrhotic patients are not entirely known, but they may include impaired synthesis in total cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol, as well as increased levels of proinflammatory cytokines and circulating endotoxin (e.g., lipopolysaccharide)[25-27]. The effects of corticosteroid therapy on cirrhotic patients with septic shock and AI are controversial, some studies reporting favorable results[12-14,28], while a recent randomized control study[29] has shown no benefit.

This review aims to summarize the existing published data regarding all aspects of AI prevalence, diagnosis and treatment in patients with liver cirrhosis.

PHYSIOLOGY OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS: A SHORT REVIEW

Cortisol is the main glucocorticoid secreted by the adrenal cortex under the control of adrenocorticotropic hormone (ACTH) which is released from the pituitary gland. The stimulus for ACTH release is corticotropin-releasing hormone (CRH) secreted by the paraventricular nuclei of the hypothalamus. Among factors influencing cortisol synthesis and production (diurnal rhythm of ACTH secretion, negative feedback by cortisol), stress plays the most important role. During stress and severe illness, activation of the hypothalamic-pituitary-adrenal (HPA) axis by the action of cytokines and other factors results in increased secretion of CRH, which will stimulate the production of ACTH and, consequently, increased release of cortisol into the circulatory system[30]. Cortisol is an essential component of the global adaptation to stress, contributing to the maintenance of cellular and organ homeostasis. Adequate levels of cortisol are absolutely necessary to increase cardiac output and vascular tonus, and to decrease proinflammatory cytokines (IL-1, IL-6, TNF-α) released[31,32] in order to overcome critical illness.

Over 90% of circulating cortisol is bound to corticosteroid-binding-globulin (CBG) (also called transcortin) and albumin, with less than 10% in the free biologically active form[33]. CBG is the predominant binding site (85%), with albumin binding smaller amounts of circulating cortisol. During severe sepsis, CBG levels fall, determining a higher percentage of free cortisol[34]. Hypoalbuminemia, frequently present in cirrhotic patients, has also been suggested to increase the free cortisol fraction[35,36]. Approximately 80% of circulating cortisol is synthesized both at rest and during stress from plasma cholesterol (particularly in the form of HDL cholesterol) and this could be relevant in patients with liver cirrhosis where cholesterol is low and may limit the synthesis of cortisol[26]. In the liver, cortisol is converted to its inactive metabolite cortisone by the enzyme 11β - hidroxysteroid dehydrogenase. After diffusion across the cell membrane, cortisol binds to glucocorticoid receptor and translocates into the nucleus of the cell[37] where its effects are exerted (increased vascular tonus and cardiac output, protein catabolism, lipolysis, hyperglycemia, and decreased cytokine production)[38]. These effects of cortisol are beneficial in critical illness, and several studies have shown that corticosteroid therapy is beneficial in patients with severe sepsis or septic shock[12-14,39,40]. As adrenal glands do not store cortisol, this must urgently be synthesized from its precursor, cholesterol, under any conditions of stress. In cirrhotic patients there is a low substrate (HDL cholesterol) for the synthesis of cortisol, favoring AI in conditions of stress[26].

PATHOGENESIS

Mechanisms leading to AI in liver cirrhosis remain largely unknown, although some hypotheses such as endotoxemia, decreased levels of apolipoprotein A-1, HDL cholesterol and LDL cholesterol, increased levels of proinflammatory mediators, structural damage to the adrenal gland due to infarction or hemorrhage, bacterial translocation of enteric organisms, "exhaustion" of the adrenal cortex, and glucocorticoid resistance have been suggested[12,41-49]. Many (if not all) of these pathophysiologic mechanisms are also involved in the genesis of AI in critically ill patients with sepsis[50-56].

As we have mentioned, cholesterol is the main source of steroidogenic substrate in the adrenal gland[26,57]. Several studies reported a significant decrease in the level

of serum HDL in cirrhotic patients which was related to the severity of the disease[12,26,47]. Furthermore, increased levels of circulating endotoxin (lipopolysaccharide) and TNF-α inhibit cortisol synthesis, limiting the delivery of HDL cholesterol to the adrenal gland[58-60]. In addition to this, TNF-α, IL-1 and IL-6 decrease hepatocyte synthesis of apolipoprotein A-1[58], the major component of HDL cholesterol. The lack of substrate for steroidogenesis will eventually lead to the so-called "adrenal exhaustion syndrome"[42] which contributes to AI in cirrhotic patients.

Besides low levels of serum total cholesterol, HDL-cholesterol and LDL-cholesterol, other factors may play a definite role in the pathogenesis of AI in patients with liver cirrhosis. Thus, coagulopathy (frequent in liver cirrhosis) may cause adrenal hemorrhage and infarction leading to structural damage of the adrenal gland[5], resulting in AI. Systemic inflammation is common in cirrhotic patients[61]. Bacterial translocation of enteric organisms has been demonstrated in patients with advanced liver cirrhosis[41,62].

A high prevalence of AI reported in patients with stable cirrhosis[15-19,63], similar to that reported in cirrhosis complicated by sepsis/septic shock, suggests that AI may be a feature of liver disease *per se*, with a different pathogenesis from that occurring in septic shock. These findings are consistent with the observations of Marik *et al*[12] who put forward the term hepato-adrenal syndrome in order to define AI in patients with advanced liver disease.

DIAGNOSIS

Diagnosis of AI made on clinical grounds in critically ill cirrhotic patients is impossible because of the lack of typical addisonian features[5,13]. Hypotension refractory to vasopressors and fluid resuscitation is the most important clinical sign in such patients[52]. Therefore, the diagnosis of AI in patients with liver cirrhosis is based on the following laboratory tests.

Standard dose

Measurement of serum total cortisol, either at baseline or following stimulation by the standard dose-short synacthen test (SD-SST) or low dose-short synacthen test (LD-SST). Baseline serum total cortisol levels under 414 nmol/L[8,13,20,64-66], < 250 nmol/L[45] or < 138 nmol/L[67] have been used to define AI in different studies. The following thresholds were used to diagnose subnormal response to SD-SST or LD-SST: (1) a peak cortisol level (defined as the highest cortisol concentration after synacthen stimulation) < 690 nmol/L[16], < 552 nmol/L[12], < 500 nmol/L[14,15,18,45], < 442 nmol/L[17]; and (2) a delta cortisol (defined as the difference between peak and basal cortisol) less than 250 nmol/L[8,13,15-20,45,64-67].

As one can easily see, there are differences in the thresholds of serum total cortisol used to define AI in published studies, which may explain significant discrepancies in the prevalence of AI in cirrhotic patients. Moreover, the diagnosis of AI based on serum total cortisol in patients with cirrhosis may be inaccurate due to changes in serum concentrations of CBG and albumin (both synthesized in the liver) which are usually low[68-70]. It has been already shown that low levels of CBG and albumin lead to overestimation of the diagnosis of AI[45,67]. As we have mentioned before, over 90% of serum circulating cortisol is bound to CBG and albumin, with less than 10% in the free form. Standard laboratory assays of serum total cortisol measure the bound plus free fractions. This means that a decrease in the binding protein levels, as it often happens in cirrhosis, will reduce serum total cortisol, affecting the interpretation of SD-SST/LD-SST[35,44], and this may lead to the overestimation of AI in cirrhotic patients[45]. However, most of the studies evaluating adrenal function in critically ill patients with liver cirrhosis still rely on the measurement of serum total cortisol, both at baseline and after stimulation.

Serum free cortisol assays are considered the most reliable method to assess adrenal function in critically ill patients[71]. There are several methods used to measure serum free cortisol (gel filtration, ultrafiltration, equilibrium dialysis)[72], all of them expensive and inconvenient for routine clinical practice[73]. In patients with liver cirrhosis, the serum free cortisol level is not altered by a reduced concentration of CBG and albumin[74] and it therefore appears to be a more appropriate marker for assessing adrenal function in such patients[44,74]. Some studies reported significant differences in diagnosis of AI using serum total cortisol and free cortisol criteria in cirrhotic patients with septic shock[75] or in those with stable cirrhosis[15], while others found that assessing serum free cortisol had limited additive diagnostic value over serum total cortisol[76]. Serum free cortisol levels under 50 nmol/L at baseline or less than 86 nmol/L after synacthen stimulation are suggestive for the diagnosis of AI (in critically ill patients)[35], although the reference range for baseline values in healthy subjects varies from 8-25 nmol/L[71] to 12-70 nmol/L[44,77].

Due to the limitations of available assays to estimate serum free cortisol, surrogate markers may be used, such as Coolens equation "$U^2 \times K (1 + N) + U [1 + N + K (G - T)] - T = 0$", where T is total cortisol, G is CBG, U is unbound cortisol, K is the affinity of CBG for cortisol at 37 ℃ and N is the ratio of albumin-bound to unbound cortisol[68], free cortisol index (FCI) (serum total cortisol concentration divided by CBG level)[78], and salivary cortisol[71,79]. However, Coolens equation and FCI do not take into account the concentration of low serum albumin and CBG frequently present in cirrhotic patients and, therefore, both surrogates may not be suitable to estimate serum free cortisol in such patients[69-71]. By contrast, salivary cortisol, regardless of serum binding protein levels, correlates well with free cortisol levels[71,79]. Basal value of salivary cortisol < 1.8 ng/mL or a concentration after stimulation (SD-SST) < 12.7 ng/mL, an increment < 3 ng/mL[45] or a peak serum free cortisol < 33 nmol/L[15] are suggestive of AI. However, there are

significant variations in normal salivary cortisol values reported by different studies[74]. Other limits of salivary cortisol are represented by oral candidiasis, low salivary flow, and contaminated salivary samples from gingival bleeding, common in cirrhotic patients[44].

SD-SST

SD-SST measures total serum cortisol at baseline and 60 min after an intravenous injection of 250 μg of synthetic ACTH. Currently, there are two corticotropic analogues that can be used, namely tetracosactrin (synacthen, Novartis Pharma AG, Basel, Switzerland) and cosyntropin (Cortrosyn, Amphastar Pharmaceuticals, Rancho Cucamonga, CA, United States). Using a supraphysiological dose of 250 μg of corticotropin (which results in approximately 100 times higher than normal maximal stress ACTH levels)[17], SD-SST is not a "physiological test"[17,80]. In the context of critical illness, AI was defined by the International Task Force[6] as a delta cortisol of < 250 nmol/L (< 9 μg/dL) after SD-SST or a random serum total cortisol of < 276 nmol/L (< 10 μg/dL). There is no consensus on the diagnostic criteria of AI in cirrhotic patients, although a delta cortisol under 250 nmol/L has been used by most studies to define AI in such patients[81].

LD-SST

LD-SST uses 1 μg of synacthen given intravenously, and serum cortisol measured after 20 and 30 min (the latter time-point is mostly used). The normal response is a serum cortisol level > 500 nmol/L (> 18 μg/dL)[49]. In a meta-analysis[82] comprising the diagnostic value of SD-SST and LD-SST for diagnosing AI, LD-SST was found to be superior, contrary to another meta-analysis[83] which reported similar operative characteristics for both tests. LD-SST seems to be a more physiological and sensitive test than SD-SST for the diagnosis of AI, and appropriate for use in non-critically ill cirrhotic patients[49].

Insulin-induced hypoglycemia test

Insulin-induced hypoglycemia test (IIHT) has been considered to be the gold standard to evaluate the HPA axis. The test uses injection of 0.15 IU/kg regular insulin to achieve blood glucose less than 40 mg/dL or until symptoms of hypoglycemia develop. Blood samples are taken before and at 15, 30, 45, 60, 90 min post-stimulation. Peak cortisol cut points between 500 and 550 nmol/L (18-20 μg/dL) are used for the diagnosis of adrenal sufficiency. This test is contraindicated in patients with cardio- or cerebrovascular diseases and convulsive disorders. In addition, the IIHT is unpleasant for the patients and therefore it has been replaced by alternative tests (LS-SST, SD-SST) for evaluating the HPA axis[84].

Corticotrophin-releasing hormone test

Corticotrophin-releasing hormone test (CRHT) evaluates the entirety of the HPA axis. Blood samples for the measurement of ACTH and cortisol are taken at baseline and at 15, 30, 45 and 60 min after an intravenous injection of 1 μg/Kg of CRH. Although CRHT is free of serious side effects, it is both difficult and costly and therefore it has been used in few studies in liver disease.

To conclude, in the absence of a gold standard test, SD-SST remains the most used test to assess the adrenal function in critically ill cirrhotic patients, while LD-SST seems to be more appropriate in those with stable cirrhosis. At present, serum free cortisol and salivary cortisol are the most accurate methods for the diagnosis of AI in cirrhotic patients, but cannot be used in routine clinical practice. The use of salivary cortisol needs to be validated. As diagnosis of AI in cirrhotics is of major clinical importance, there is an urgent need for a consensus as to which is the most appropriate diagnostic test of AI in such category of patients.

PREVALENCE AND EXISTING EVIDENCE

Initial reports on AI in liver cirrhosis were followed by multiple studies (Tables 1 and 2) and, recently, by excellent systematic reviews[43,44,46,49,81]. There are significant discrepancies between studies on the prevalence of AI in patients with liver cirrhosis, mainly because of the different tests used for diagnosis of adrenal dysfunction and the criteria applied to define AI. Thus, the prevalence of AI varies between critically ill cirrhotic patients (10%-87%; Table 1), those with stable cirrhosis (7%-83%; Table 2), and patients with liver transplant (61%-92%; Table 1). Overall, several published studies have reported a high prevalence of AI both in critically and non-critically ill cirrhotic patients[17,29,63,64,69,85] as well as in those who had received liver transplant[12].

Critically ill patients with liver cirrhosis

Almost all studies that included critically ill patients with liver cirrhosis[8,13,20,29,64-66,74,85] used SD-SST for the diagnosis of AI and only two performed LD-SST[12,16]. With SD-SST, the reported prevalence of AI in critically ill cirrhotics varied between 10%[74] and 87%[85], while with LD-SST, the prevalence range was between 33%[12] and 60%[16].

Harry *et al*[14] reported a prevalence of AI (defined as peak cortisol levels less than 500 nmol/L) of 69% in critically ill cirrhotic patients requiring vasopressor support. In a prospective study including 25 cirrhotic patients with severe sepsis, Fernández *et al*[13] reported a very high incidence of AI (68%) using SD-SST and defining AI either as baseline serum total cortisol level less than 414 nmol/L or a delta cortisol lower than 250 nmol/L in those with a baseline concentration below 966 nmol/L. The AI prevalence rate was correlated with the severity of liver disease (76% Child-Pugh C *vs* 25% Child-Pugh B).

SD-SST was also used to evaluate adrenal function in a prospective study which included 101 critically ill patients with cirrhosis and severe sepsis[8]. Authors found that 51% of their patients met the criteria for AI (defined as baseline serum total cortisol values under 414 nmol/L

Table 1 Prevalence of adrenal insufficiency in critically ill patients with liver cirrhosis

Ref.	No. of patients (type of cirrhosis)	Diagnosis and definition of AI	Prevalence of AI
Harry *et al*[14]	20 (ALF/CLD)	SD-SST: Peak cortisol < 500 nmol/L[1]	69%
Marik *et al*[12]	340	LD-SST: Peak cortisol < 552 nmol/L or	72%
	(ALF: 24)	random cortisol level < 414 nmol/L in non-stressed patients or	33%
	(CLD: 146)	random cortisol level < 552 nmol/L in stressed patients	66%
	(recent LT: 119)		92%
	(remote LT: 51)		61%
Tsai *et al*[8]	101 (cirrhosis+ severe sepsis)	SD-SST: Baseline cortisol < 414 nmol/L or delta cortisol < 250 nmol/L if baseline cortisol between 414 and 938 nmol/L	51%
Fernandez *et al*[13]	25 (cirrhosis + septic shock)	SD-SST: Baseline cortisol < 414 nmol/L or delta cortisol < 250 nmol/L if baseline cortisol between 414 and 966 nmol/L	68%
Thierry *et al*[64]	14 (cirrhosis + septic shock)	SD-SST: Baseline cortisol < 414 nmol/L; delta cortisol < 250 nmol/L	77%
du Cheyron *et al*[65]	50 (critically ill cirrhosis)	SD-SST: Baseline cortisol < 414 nmol/L; delta cortisol < 250 nmol/L if baseline cortisol between 414 and 938 nmol/L	82%
Vasu *et al*[86]	24 (critically ill cirrhotics)	SD-SST: Definition of AI was not reported	62%
Arabi *et al*[29]	75 (cirrhosis + septic shock)	SD-SST: Delta cortisol < 250 nmol/L	76%
Mohamed *et al*[85]	15 (cirrhosis+septic shock)	SD-SST: Definition of AI was not reported	87%
Thevenot *et al*[74]	30 (cirrhosis + sepsis)	SD-SST: Peak serum total cortisol < 510 nmol/L	10%
Acevedo *et al*[89]	166 (decompensated cirrhosis)	SD-SST: Delta cortisol < 250 nmol/L	26%
Graupera *et al*[20]	37 (severe acute bleeding)	SD-SST: Baseline cortisol < 414 nmol/L and/or delta cortisol < 250 nmol/L	38%
Triantos *et al*[16]	20 (cirrhosis with variceal bleeding)	SD-SST: Baseline cortisol < 276 nmol/L or delta cortisol < 250 nmol/L	30%
		LD-SST: Peak serum cortisol < 690 nmol/L or a delta cortisol < 250 nmol/L	60%
El Damarawy *et al*[66]	45 (cirrhosis with septic shock or HRS, cirrhosis without septic shock or HRS)	SD-SST: Baseline cortisol < 414 nmol/L or delta cortisol < 250 nmol/L in patients with baseline cortisol < 966 nmol/L	73%

[1]To convert serum total cortisol concentrations from nanomoles per liter to micrograms per deciliter divide by 27.59[79]. ALF: Acute liver failure; CLD: Chronic liver disease; HRS: Hepatorenal syndrome; LT: Liver transplant; AI: Adrenal insufficiency; SD-SST: Standard dose short synacthen test; LD-SST: Low dose short synacthen test.

or delta cortisol lower than 250 nmol/L with a baseline value between 414 and 938 nmol/L) which was related to disease severity [Child-Pugh and model for end-stage liver disease (MELD) scores] and increased mortality. Recently, Arabi *et al*[29], using the same test (SD-SST) and definition for AI (delta cortisol < 250 nmol/L) in a similar group of critically ill patients (cirrhosis with septic shock) reported an even higher AI prevalence rate (76%).

The SD-SST test was also used in several other studies to assess adrenal function in critically ill cirrhotic patients[64-66,74,85,86].

Adrenal function has also been evaluated by SD-SST in cirrhotic patients with variceal bleeding[16,20]. Graupera *et al*[20] reported AI prevalence (defined as baseline serum cortisol < 414 nmol/L or delta cortisol < 250 nmol/L) in 38% of bleeding patients. AI was associated with increased risk of failure to control bleeding and lower survival rate at 6 wk. In a prospective observational study on 20 cirrhotic patients with variceal bleeding and 60 with stable cirrhosis, Triantos *et al*[16] reported an AI prevalence rate (defined as basal cortisol < 276 nmol/L or delta cortisol < 250 nmol/L following SD-SST) of 30% (similar to that in stable cirrhosis); with the use of LD-SST, AI prevalence (defined as a peak cortisol < 690 nmol/L or a delta cortisol < 250 nmol/L) was significantly higher in bleeders (60%) than in stable cirrhotics (48%).

LD-SST was also previously used by Marik *et al*[12] to evaluate adrenal function in 340 critically ill patients with liver disease (24 with fulminant hepatic failure, 146 critically ill cirrhotics, 51 with remote LT, and 119 having recently undergone LT). AI was defined as having a random cortisol level of < 552 nmol/L in highly stressed patients (hypotension, hepatic failure, respiratory failure) and a random cortisol level of < 414 nmol/L or a 30 min post LD-SST level of < 552 nmol/L in all other patients. Out of 340 patients studied, 245 (72%) met the criteria for AI (33% fulminant hepatic failure, 66% critically ill cirrhotics, 61% remote LT, 92% recent LT).

Non-critically ill cirrhotics

AI is also common in patients with stable liver cirrhosis (Table 2). However, as in critically ill cirrhotic patients, AI prevalence rate in those with stable liver cirrhosis varies significantly, depending on the diagnostic test used.

In a prospective study, Tan *et al*[15] evaluated adrenal function in 43 clinically stable cirrhotic patients. All patients underwent SD-SST, and AI was defined by delta cortisol < 250 nmol/L or a peak total cortisol < 500 nmol/L, or a peak serum free cortisol < 33 nmol/L. The prevalence of AI was 47% using delta cortisol < 250 nmol/L, 39% using peak total cortisol < 500 nmol/L, and 12% with serum free cortisol < 33 nmol/L. This study clearly shows that the reported prevalence of AI depends largely on the diagnostic test used and criteria for defining AI.

Galbois *et al*[45] have evaluated adrenal function in 88 patients hospitalized for complications of cirrhosis without bleeding and shock. Salivary and serum total cortisol were assessed 60 min before and after stimulation with SD-SST in all patients. Serum free cortisol was estimated

Table 2 Prevalence of adrenal insufficiency in patients with liver cirrhosis, not critically ill

Ref.	No. of patients (type of cirrhosis)	Diagnosis and definition of AI	Prevalence of AI
McDonald *et al*[69]	38 (stable cirrhosis)	IIHT: Reduction in maximal increments of plasma cortisol	64%
		SD-SST: Reduction in maximal increments of plasma cortisol	39%
Zietz *et al*[112]	52 (stable cirrhosis)	CRHT: Peak cortisol < 550 nmol/L or an increase < 250 nmol/L[1]	58%
		rise of plasma ACTH < twice the baseline	42%
Sigalas *et al*[87]	47 (stable cirrhosis)	SD-SST: Baseline cortisol < 250 nmol/L and delta cortisol < 250 nmol/L	36%
Alessandria *et al*[88]	25 (cirrhosis and ascites)	SD-SST: Delta cortisol < 250 nmol/L	36%
Jang *et al*[63]	18 (stable cirrhosis)	SD-SST: Baseline cortisol < 414 nmol/L delta cortisol < 250 nmol/L	83%
Acevedo *et al*[19]	198 (10 compensated and	SD-SST: Baseline cortisol < 414 nmol/L	64%
	188 decompensated cirrhosis)	delta cortisol < 250 nmol/L	27%
Galbois *et al*[45]	88 (stable cirrhosis)	SD-SST: (1) Serum total cortisol: Baseline cortisol < 250 nmol/L or peak cortisol < 500 nmol/L or delta cortisol < 250 nmol/L	33%
		(2) Salivary cortisol: Basal salivary cortisol < 1.8 ng/mL or post-stimulation values < 12.7 ng/mL or increase values < 3 ng/mL	9%
Tan *et al*[15]	43 (stable cirrhosis)	SD-SST: Peak total cortisol < 500 nmol/L;	39%
		delta cortisol < 250 nmol/L;	47%
		peak plasma free cortisol < 33 nmol/L;	12%
		any set of criteria	58%
Thevenot *et al*[67]	95 (stable cirrhosis)	LD-SST: Baseline cortisol < 138 nmol/L;	7%
		< 440 nmol/L after stimulation;	19%
		⩽ 500 nmol/L after stimulation;	27%
		delta cortisol < 250 nmol/L	49%
Fede *et al*[17]	101 (stable cirrhosis)	LD-SST: Peak serum cortisol < 500 nmol/L;	38%
		peak serum cortisol < 442 nmol/L;	29%
		delta cortisol < 250 nmol/L	60%
Triantos *et al*[16]	60 (stable cirrhosis)	SD-SST: Peak serum cortisol < 500 nmol/L	30%
		LD-SST: Peak serum cortisol < 500 nmol/L	48%
Mohamed *et al*[85]	15 (stable cirrhosis)	SD-SST: Definition of AI was not reported	53%
Risso *et al*[18]	85 (cirrhosis with ascites, without sepsis or shock)	SD-SST: Delta cortisol < 250 nmol/L and/or peak cortisol < 500 nmol/L	39%
Vincent *et al*[73]	26 (liver impairment)	SD-SST: Serum total cortisol < 550 nmol/L;	46%
		free cortisol index < 12	13%

[1]To convert serum total cortisol concentrations from nanomoles per liter to micrograms per deciliter divide by 27.59[79]. AI: Adrenal insufficiency; SD-SST: Standard dose short synacthen test; LD–SST: Low dose short synacthen test; CRHT: Corticotropin-releasing hormone test; IIHT: Insulin-induced hypoglycemia test; ACTH: Adrenocorticotropic hormone.

from serum total cortisol and CBG levels using Coolens' formula[68]. The following definitions of AI were used by the authors: (1) according to serum total cortisol assays: baseline < 250 nmol/L, or a peak total cortisol < 500 nmol/L, or delta cortisol < 250 nmol/L; (2) according to salivary cortisol assays: baseline < 1.8 ng/mL, or an increase < 3 ng/mL or a concentration < 12.7 ng/mL after stimulation. The results indicated a significant difference in AI prevalence depending on the test used: 33% when serum total cortisol was considered *vs* 9.1% using salivary cortisol.

Another study performed by Thevenot *et al*[74] has demonstrated that assessment of adrenal function with measurements of serum total cortisol overestimated AI prevalence in cirrhotic patients. In this study, baseline and post-synacthen serum total cortisol, serum free cortisol and salivary cortisol concentrations were measured in 125 cirrhotic patients (95 non-septic, 30 septic). AI was defined as serum total cortisol < 510.4 nmol/L after SD-SST. AI was found in nine patients (7.2%) (6 non-septic; 3 septic) and restricted to cirrhotics with Child-Pugh C. Serum total cortisol concentrations, CBG and albumin levels significantly decreased in non-septic patients as liver function deteriorated (from Child-Pugh A to C). Cirrhotic patients with or without AI had similar basal serum free cortisol and salivary cortisol levels. As the serum total cortisol level overestimated the prevalence of AI in cirrhotic patients, and serum free cortisol is not suitable for routine laboratory use, authors concluded that measurement of salivary cortisol is a useful approach in such patients. The same group of investigators[67] analyzed only the 95 hemodynamically stable cirrhotic patients from the previously mentioned study, who underwent a LD-SST. The serum total cortisol and serum free cortisol concentrations were measured 30 min before and after LD-SST. AI was defined as: (1) basal serum total cortisol < 138 nmol/L and < 440 nmol/L after stimulation; (2) serum total cortisol < 500 nmol/L after stimulation; and (3) cortisol increment < 250 nmol/L. AI prevalence rates varied significantly according to the threshold used: 7.4 % with basal serum total cortisol, 19% using serum cortisol < 440 nmol/L, 27.4 % with serum cortisol < 500 nmol/L, and 49.4% with delta cortisol. Serum free cortisol levels before and after LD-SST stimulation were higher in the more severe cirrhotic patients regardless of CBG and albumin concentrations, and directly associated with the risk of non-transplant-related mortality in hemodynamically stable patients with cirrhosis.

In opposition to the above mentioned studies, recently, in a prospective study, Molenaar *et al*[76], using SD-SST, assessed the value of free *vs* total cortisol levels while evaluating AI in 49 septic and 63 non-septic patients with treatment-insensitive hypotension and found that total cortisol correlated with free cortisol during critical illness. Moreover, in sepsis, hypoalbuminemia did not affect total and free cortisol, contrary to the findings of other published studies[45,67].

Others, using SD-SST or LD-SST to diagnose adrenal dysfunction in patients with stable liver cirrhosis reported high AI prevalence rates[16-19,63,69,73,85,87,88]. Fede *et al*[17] reported an AI prevalence of 38% in 101 patients with stable cirrhosis (absence of infections or hemodynamic instability). AI, defined as a peak serum total cortisol level < 500 nmol/L after LD-SST, was correlated with the severity of liver disease graded according to Child-Pugh or MELD scores.

Using SD-SST in 85 cirrhotics with ascites but without sepsis, Risso *et al*[18] reported AI (delta cortisol < 250 nmol/L and/or peak cortisol < 500 nmol/L) in 39% of patients.

Vincent *et al*[73] evaluated adrenal function by SD-SST in 26 patients with liver impairment. Authors defined AI as serum total cortisol < 550 nmol/L or FCI < 12. Three patients (13%) met both criteria, 12 patients (46%) had a serum total cortisol < 550 nmol/L but an FCI > 12. When serum total cortisol was used, 46% of patients had AI, while when using FCI only 13% fulfilled the criteria for AI. Authors suggested that FCI is better suited for the evaluation of AI in patients with liver impairment.

Acevedo *et al*[19], using SD-SST, evaluated the prevalence of AI in 198 patients with liver cirrhosis [10 with compensated, 188 with decompensated cirrhosis and complications (hepatic encephalopathy, spontaneous bacterial peritonitis, ascites, gastrointestinal bleeding, hepatorenal syndrome)]. AI defined as basal serum total cortisol < 414 nmol/L was found in 64% of patients, and only in 27% when delta cortisol < 250 nmol/L was used, with no differences between compensated and decompensated cirrhosis. The same group of researchers evaluated the prevalence and prognostic value of AI in 166 patients with advanced cirrhosis (no severe sepsis or septic shock)[89]. AI, defined as delta cortisol < 250 nmol/L after SD-SST, was found in 26% of patients. Those with AI had a higher degree of circulatory dysfunction, greater prevalence of systemic inflammatory response syndrome, increased probability to develop severe infections, and higher hospital mortality rates than patients without AI.

AI after LT

AI has been reported both early as well as late after LT[12,21-23,90].

With LD-SST, Marik *et al*[12] found that 92% of 119 patients undergoing recent LT and maintained on steroid-free immunosuppressive regimens had AI. The steroid-free immunosuppressive regimen may expose patients undergoing LT to an increased risk for AI, while the use of steroids intra and postoperatively in LT may reduce such a risk or mask an AI[46]. Furthermore, LD-SST is not recommended for the diagnosis of AI in high-stress conditions like LT[6] as it may lead to an overestimated AI prevalence in such patients.

Toniutto *et al*[21], using SD-SST, reported an AI prevalence rate of 26% in 87 patients having received LT for end-stage liver disease and maintained on prolonged immunosuppressive treatment.

Patel *et al*[90] reported significantly reduced requirements for fluid, vasopressors, invasive ventilation, and renal replacement therapy, and intensive care unit stay for patients undergoing LT who received 1000 mg methylprednisolone prior to the liver graft reperfusion.

TREATMENT

Cortisol has several beneficial effects such as an increase of the vascular tonus and cardiac output, enhancement of catecholamine responsiveness, inhibition of the production of nitric oxide, modulation of cytokine production in septic shock[32,91-97], but the effects of corticosteroid therapy in sepsis, severe sepsis and septic shock remain, however, controversial. Thus, a significant reduction in mortality rate with hydrocortisone therapy in patients with septic shock has been reported in several studies and meta-analyses[6,28,39,98-101], while others have shown no effect on the 28-d mortality rate[14,29,102]. Both doses and duration of corticosteroid therapy vary significantly in published studies[6,28,39,40,102,103]. Thus, some used a daily dose of hydrocortisone (or equivalent) of 200-300 mg ("low-dose", also called "physiologic-dose" or "stress-dose")[3,28,39,98,100-105] while others used a "supra-physiologic" dose (> 300 mg)[98,106-108].

None of the early studies using high doses of corticosteroids for short courses reported any benefit[98,106-108], while more recent studies using a "physiologic-dose" for longer durations have shown a significant reduction in vasopressor agents requirement and in intensive care unit length of stay, greater shock resolution, and decreased mortality[6,28,39,98,100,104,105,109-111]. A randomized, double-blind placebo controlled trial, CORTICUS (Corticosteroid Therapy of Septic Shock)[102] including 499 patients with septic shock randomized to hydrocortisone (50 mg intravenously every 6 h for 5 d, followed by 50 mg intravenously every 12 h for 3 d, and then by 50 mg daily for 3 d) or placebo, concluded that there was no benefit in terms of mortality, although steroid administration was associated with a greater shock reversal, but also with a higher incidence of episodes of new infections. On the other hand, Annane *et al*[28] in a randomized, double-blind controlled trial have found that the administration of hydrocortisone (50 mg intravenously every 6 h) and oral fludrocortisone (50 μg once daily) in patients with refractory septic shock and AI (delta cortisol < 250 nmol/L) resulted in a 30% decrease in 28-d mortality. It should be mentioned that consensus statements from an international task force[6] recommended corticosteroid therapy

Table 3 Published studies on corticosteroid therapy in patients with liver cirrhosis

Ref.	No. of patients (type of cirrhosis)	Study design	Steroid dose	Outcomes
Harry *et al*[14]	20 (ALF or ACLF)	Retrospective	Hydrocortisone 300 mg/d	Reduction in vasopressor doses, but higher incidence of infection and no survival benefit
Marik *et al*[12]	140 (ALF or CLD)	Not RCT	Hydrocortisone 300 mg/d	Reduction in the dose of norepinephrine at 24 h, and lower mortality rate increased survival
Fernandez *et al*[13]	17 (cirrhosis and septic shock)	Prospective but not RCT	Hydrocortisone 200 mg/d	Significant increase in shock resolution and high hospital survival rate
Arabi *et al*[29]	39 (cirrhosis and septic shock)	RCT	Hydrocortisone 200 mg/d	Reduction in vasopressor doses and higher rates of shock reversal, but no benefit in 28 d mortality, increase in gastrointestinal bleeding and shock relapse

ALF: Acute liver failure; ACLF: Acute-on-chronic liver failure; CLD: Chronic liver disease; RCT: Randomized controlled trial.

(intravenous hydrocortisone 200-300 mg/d in four divided doses for a week before tapering slowly) in patients with vasopressor-dependant septic shock.

Like in patients with severe sepsis/septic shock with other causes than liver cirrhosis, as mentioned above, the effects of steroid therapy in cirrhotic patients with AI remain controversial, some studies reporting beneficial results[12-14] while a recent randomized control study[29] has shown no benefit (Table 3).

Harry *et al*[14] evaluated the effects of stress doses of hydrocortisone in a retrospective comparative study including 40 patients. Twenty patients received hydrocortisone (300 mg/d) for 4-5 d. In patients with acute-on-chronic liver failure requiring norepinephrine support, the results showed a reduction in vasopressor doses, but no survival benefit; moreover, corticosteroid therapy was associated with a significant increase in infections.

Another study, carried out by Marik *et al*[12] evaluated the effect of 300 mg/d hydrocortisone given intravenously in vasopressor-dependant patients with acute or chronic liver disease. In patients with AI, treatment with hydrocortisone was associated with a significant reduction of the norepinephrine dosage at 24 h and with a lower mortality ($P = 0.02$), whereas in those patients without AI hydrocortisone did not affect the norepinephrine dose.

Fernández *et al*[13], in a prospective but non-randomized study have evaluated adrenal function by SD-SST and the effects of low-dose hydrocortisone in 25 patients with advanced cirrhosis and septic shock. Patients with AI received intravenous hydrocortisone (50 mg every 6 h) and results were compared with those obtained from a retrospective 50 cirrhotic patients with septic shock in whom adrenal function was not investigated and who did not receive corticosteroid therapy. Results showed that hydrocortisone therapy was associated with a significant increase in shock resolution and hospital survival rate. Authors suggested that all cirrhotic patients with AI should be treated with hydrocortisone.

Recently, Arabi *et al*[29] in a randomized controlled trial, have shown that low dose hydrocortisone therapy in cirrhotic patients with septic shock had a significant reduction in vasopressor doses and higher rates of shock reversal, but it did not reduce mortality and was associated with an increase in adverse effects (gastrointestinal bleeding) and shock relapse.

Based on the above mentioned studies, there are still several unsolved problems and questions awaiting answers. Thus, re-evaluation of both doses and duration of corticosteroid therapy is necessary. Obviously, further prospective randomized clinical studies are needed to assess the effect of corticosteroid therapy in critically ill cirrhotic patients with AI.

CONCLUSION

AI occurs frequently in patients with liver cirrhosis both during critical illness and in stable disease. Studies, however, do not agree on the prevalence of AI in cirrhotic patients, mostly because of the different criteria and the methodology used to define AI. Diagnosis of AI in patients with liver cirrhosis remains controversial (particularly in those critically ill) as all diagnostic tests proved their limitations. Pathogenesis of AI in liver cirrhosis is still unknown, although decreased levels of cholesterol (mainly HDL cholesterol) and increased levels of pro-inflammatory cytokines and circulating endotoxin have been put forward. Some data suggest that AI may be a feature of cirrhosis *per se*, with a pathogenesis subtly different from that occurring in septic shock from other causes. Yet, there is still controversy in what concerns treatment with corticosteroids, although some cirrhotic patients with vasopressor resistant shock may benefit. However, further prospective, randomized clinical trials are necessary to assess the effect of corticosteroid therapy in critically ill patients with cirrhosis.

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REVIEW

Obesity and cholangiocarcinoma

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Abstract

It is estimated that about half of the population in developed countries are either overweight or obese. In some developing nations obesity rates have increased to surpass those seen in Western countries. This rate increase in obesity has many implications as obesity has been associated with numerous negative health effects including increased risks of hypertension, diabetes, cardiovascular disease, stroke, liver disease, apnea, and some cancer types. Obesity is now considered to be one of the major public health concerns facing the society. Cholangiocarcinomas (bile duct cancers) are malignant tumors arising from cholangiocytes inside or outside of the liver. Although cholangiocarcinomas are relatively rare, they are highly lethal. The low survival rate associated with cholangiocarcinoma is due to the advanced stage of the disease at the time of diagnosis. Prevention is therefore especially important in this cancer type. Some data suggest that the incidence of cholangiocarcinoma in the western world is on the rise. Increasing rate of obesity may be one of the factors responsible for this increase. Determining whether obesity is a risk factor for cholangiocarcinoma has significant clinical and societal implications as obesity is both prevalent and modifiable. This paper seeks to provide a summary of the current knowledge linking obesity and cholangiocarcinoma, and encourage further research on this topic.

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Key words: Cholangiocarcinoma; Obesity; Epidemiology; Bile ducts; Cancer

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INTRODUCTION

Obesity is increasing worldwide and affects a large portion of the population in developed[1-5] and some developing nations[6-13]. In many countries obesity and its comorbidities have become a major public health concern[1,14-16]. Recently, obesity-associated cancer has attracted great attention. Epidemiological studies have provided strong evidence that obesity can increase the incidence of many cancer types[17] including colon[18,19], breast[20,21], liver[22], endometrium[23,24], kidney[25], esophagus[26], stomach[27] and pancreas[28].

Cholangiocarcinomas (bile duct cancers) are malignant tumors arising from cholangiocytes inside or outside of the liver. Traditionally cancers arising from the cholangiocytes inside the liver were grouped together with hepatic cancers and classified as primary liver tumors, while those arising from the cholangiocytes outside the liver were grouped together with cancers of the gallbladder and the ampulla of Vater and classified as biliary cancers[29]. More recently, the term cholangiocarcinoma has been used to refer to cancers arising from cholangiocytes irrespective of their location[29].

Intrahepatic cholangiocarcinomas originate from cholangiocytes lining the small intrahepatic ductules while extrahepatic cholangiocarcinomas arise from cholangiocytes lining the larger bile ducts (right and left hepatic ducts, common hepatic duct, and common bile duct)[30]. The distinction between intrahepatic and extrahepatic cholangiocarcinomas is important, as the incidence and risk factors associated with each may be different[31-33].

In Western countries established risk factors for cholangiocarcinoma are age, sex, primary sclerosing cholangitis, and bile duct cysts. Less-established potential risk factors include cirrhosis, diabetes, obesity, alcohol, and smoking. Some data suggest that the incidence of cholangiocarcinoma in the Western world is on the rise. Increasing rate of obesity may be one of the factors responsible for this increase.

Although cholangiocarcinomas are rare in Western countries, they are highly lethal[34]. Annual mortality associated with cholangiocarcinoma is virtually identical to its incidence throughout the world, underscoring the high case fatality rate of this cancer type[35]. The low survival rate associated with cholangiocarcinoma is due to the advanced stage of the disease at the time of diagnosis[36]. Prevention is therefore especially important in this cancer type. Determining whether obesity is a risk factor for cholangiocarcinoma has significant clinical and societal implications as obesity is both prevalent and modifiable.

EPIDEMIOLOGY

Obesity prevalence

Obesity is considered to be among the most alarming health concerns in the United States. It has been estimated that each year, more than 300 000 Americans die from illnesses related to overweight and obesity[37]. The Centers for Disease Control and Prevention (CDC) defines obesity based on the body mass index (BMI). BMI is the ratio of weight in kilograms (kg) to height in meters squared (m^2). Per CDC criteria, a BMI of 18.5-25 kg/m^2 is normal, 25-30 kg/m^2 is overweight and $\geqslant$ 30 kg/m^2 is obese[38]. The most recent statistics from the National Health and Nutrition Examination Surveys using BMI as a measure of overweight and obesity indicate that 35.7% of adults 20 years or older were obese in 2009-2010[39]. Compared with the statistics for 1976-1980 this reflects a 2-fold increase in the prevalence of adult obesity[40,41]. This rate increase in obesity has many implications as obesity has been associated with numerous negative health effects including increased risks of hypertension, diabetes, cardiovascular disease, stroke, liver disease, apnea, and some cancer types possibly also cholangiocarcinoma. Therefore, as a potentially modifiable risk factor, a reduction in the prevalence of obesity among adults could have a substantial impact on morbidity, mortality, and incidence of various cancers.

Cholangiocarcinoma incidence

The available epidemiologic data on incidence and prevalence of cholangiocarcinoma are difficult to interpret because of differing classifications and diagnostic criteria across studies[29,32,42]. For instance, some studies group intrahepatic cholangiocarcinomas with liver cancers while others group extrahepatic cholangiocarcinomas with gallbladder cancers and cancers of the ampulla of Vater. Furthermore, some studies misclassify types of cholangiocarcinomas. For example, Klatskin tumors (a type of extrahepatic cholangiocarcinoma), at times, have been misclassified as intrahepatic cholangiocarcinoma[29,30]. Finally, some studies use histology as the only diagnostic criterion while others accept other forms of diagnosis such as clinical evaluation or imaging studies.

Despite these shortcomings, the available data suggest a highly variable incidence in different parts of the world, with the highest incidence in Southeast Asia and lowest in Australia[43,44]. The incidence of cholangiocarcinoma has been reported to be as high as 96 cases per 100 000 in men and 38 per 100 000 in women in northeast Thailand[43]. In Australia, the reported incidence is 0.2 per 100 000 among Australian men and 0.1 per 100 000 among Australian women[43]. In other Western countries including the Unites States the incidence is close to 1 case per 100 000 population[43]. In Western countries, approximately 80% of cholangiocarcinomas are extrahepatic[45]. Differing exposure to risk factors is thought to account for the varying geographic incidences, with parasitic infections and hepatolithiasis being more prevalent in Asia[46-48].

Recent data show that the incidence of intrahepatic cholangiocarcinoma is increasing around the world, while the incidence of extrahepatic cholangiocarcinoma has been largely stable or even decreasing[32,43,49]. Although the exact underlying mechanism for this discrepancy is not known, it suggests that intrahepatic and extrahepatic cholangiocarcinomas may be biologically different[32,49,50].

Finally, there is evidence suggesting under-diagnosis of cholangiocarcinoma. For example among patients transplanted for liver cirrhosis of various etiologies, an incidental cholangiocarcinoma was found in approximately 12% of explanted livers[51]. This suggests that the true incidence of cholangiocarcinoma may be higher than previously reported.

Obesity as a risk factor for cholangiocarcinoma

Data assessing the association of obesity and cholangiocarcinoma are limited. So far, four epidemiologic studies have tried to assess the link between obesity and cholangiocarcinoma (Table 1). Three of these studies were specifically designed to identify risk factors for cholangiocarcinoma[52-54].

One of those studies using the Surveillance, Epidemiology, and End Results database that links cancer registry data and Medicare enrollment and claims files, reported a significant association between obesity and intrahepatic cholangiocarcinoma, but not between obesity and extrahepatic cholangiocarcinoma[52]. That study however, has several limitations. The study only included patients 65 years of age and older, therefore the findings might not be generalizable to a younger population. Furthermore, because Medicare data are collected for billing rather than research purposes, completeness and accuracy of the Medicare data on prevalence of obesity and cholangiocarcinoma can be questioned. Finally, the possibility of diagnostic bias cannot be excluded because obese people with different medical conditions are more likely to undergo testing and thus have more diagnoses than other people.

Another population-based study conducted in Denmark did not find any significant association between

Table 1 Epidemiologic studies assessing obesity as a potential risk factor for cholangiocarcinoma

Ref.	Study design	Type of CC	Study findings	Study limitations
Welzel *et al*[52]	Case-control	ICC and ECC	Positive association between obesity and ICC; no association between obesity and ECC	Only included age ⩾ 65 yr; database limitations; possible diagnostic bias
Welzel *et al*[53]	Case-control	ICC	No association between obesity and ICC	ECC not included
Grange *et al*[54]	Case-control	NS	Positive association between obesity and cholangiocarcinoma	Type of CC not specified; diagnosis subject to misclassification
Oh *et al*[55]	Cohort	NS	Positive association between obesity and cholangiocarcinoma	Does not distinguish ECC and ICC; included only males; database limitations

CC: Cholangiocarcinoma; ECC: Extrahepatic cholangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma; NS: Not stated.

obesity and intrahepatic cholangiocarcinoma[53]. That study used a fairly accurate and complete national registry. Another strength of that study was histological confirmation of cholangiocarcinoma diagnosis in all cases. Extrahepatic cholangiocarcinoma, however, was not studied.

The third study, conducted in the United Kingdom by Grainge *et al*[54], found that obese patients (BMI ⩾ 30 kg/m^2) had 1.5 times the risk of cholangiocarcinoma compared with those with BMI < 25 kg/m^2. In that study the type of cholangiocarcinoma (intrahepatic versus extrahepatic) was not specified. Another limitation of that study was that the diagnosis was not based on histology and therefore subject to misclassification.

Aside from studies looking specifically to identify risk factors for cholangiocarcinoma, a South Korean population-based study tried to assess the effect of excess weight on incidence of various cancers including cholangiocarcinoma[55]. The study found that compared to the reference group (BMI 18.5-23 kg/m^2), increasing BMI was associated with an increase in the relative risk for development of cholangiocarcinoma[55]. That study, however, did not distinguish between intrahepatic and extrahepatic cholangiocarcinomas, included only males, and the database used was not inclusive of the entire population.

In summary, the epidemiologic data available on obesity as a risk factor for cholangiocarcinoma show conflicting results and are too limited to make any conclusions.

BIOLOGIC MECHANISMS FOR POSITIVE ASSOCIATION BETWEEN OBESITY AND CHOLANGIOCARCINOMA

Obesity is an excess of adipose tissue. Adipose tissue is a biologically active organ which in addition to adipocytes contains multiple other cell types such as pre-adipocytes, endothelial cells, macrophages and other immune cells[56]. Adipose tissue secretes molecules into the bloodstream, which signal to other metabolic organs or to the brain to coordinate responses to altered metabolic demands[57]. Some of these molecules, known as adipokines, have a role in modulating the risk of cancer development. Among adipokines, some of the most studied are leptin, adiponectin and pro-inflammatory molecules.

Leptin is a hormone that is primarily secreted by the adipose tissue[58]. It acts on the hypothalamus to regulate food consumption and caloric homeostasis[58,59]. Although the major physiological site of leptin action is in the central nervous system, leptin receptors are also expressed at lower levels in peripheral tissues[60]. Two studies have shown presence of leptin receptors on cholangiocytes[61,62]. Circulating serum leptin is increased in obesity, and has been suggested as a risk factor for cholangiocarcinoma[62]. A recent study showed that normal and malignant intrahepatic cholangiocytes express leptin and leptin receptors[62]. The investigators of that study also showed that leptin stimulates growth and migration, and prevents apoptosis of intrahepatic cholangiocarcinoma cells *in vitro*[62]. They also showed that genetic ablation of leptin-mediated signaling inhibits cancer development and growth in an animal model of cholangiocarcinoma[62].

Adiponectin is another adipokine that may be involved in cancer development[63]. It acts on a number of tissues to regulate glucose and lipid metabolism. Adiponectin levels are reduced in obesity. Several basic and epidemiological studies have suggested that adiponectin has antitumor effects[63,64]. Although the link between adiponectin and cholangiocarcinoma has not been studied, there is convincing evidence for an inverse association between adiponectin levels and cancer risk for several cancer types[59,63,65-67].

Several pro-inflammatory cytokines are secreted by monocytes and other immune cells that infiltrate adipose tissues in obesity[68,69]. Among these cytokines, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) have been found to have a role in development of cholangiocarcinoma[70]. A growing body of evidence suggests that the inflammatory environment in the obese state is linked to the development of cancer through various mechanisms[71]. IL-6 seems to have an important role in the pathogenesis and growth of cholangiocarcinoma and has been shown to act by both an autocrine and paracrine manner stimulating several intracellular pathways involved in survival and growth of malignant cholangiocytes[36,72-74]. TNF-α is a mediator of inflammation with actions directed towards both tissue destruction and recovery[70]. Evidence suggests that TNF-α may act as an endogenous tumor promoter in addition to its role in immune responses[75,76]. Overexpression of TNF-α receptor genes has been observed in cholangiocarcinoma associated with hepatolithiasis[75-78].

Although not specifically linked to cholangiocarcino-

ma, there are multiple other substances that have directly or indirectly been linked to cancer in general[57]. Research on the role of different substances on development and progression of different cancers including cholangiocarcinoma is evolving.

DISCUSSION

It is estimated that about half of the population in developed countries are either overweight or obese[79]. In some regions of the world overweight and obesity affects more than two-thirds of the population[80]. By 2008, an estimated 1.5 billion adults globally were overweight and 500 million adults were obese[81]. Because of such high prevalence, establishing a clear link between excess body weight and cholangiocarcinoma can have significant effect on prevention of cholangiocarcinoma. Establishing a link may also encourage further research on identification of underlying pathophysiologic mechanisms that can potentially lead to discovery of better treatment options.

Research on obesity and cholangiocarcinoma risk should be encouraged through a variety of activities, including large cooperative initiatives, database resources, epidemiologic studies, basic science studies, and increase in funding resources.

If a link between excess weight and cholangiocarcinoma is established, multiple new questions will emerge that will need to be answered. Does reversal of obesity affect the risk for cholangiocarcinoma? Are there any differences between different modes of weight reduction (calorie restriction, exercise, drugs, bariatric surgery) in terms of cholangiocarcinoma risk reduction? Can the link between obesity and cholangiocarcinoma be somehow disrupted in the absence of weight loss (which for many individuals is a difficult task)?

Answers to these and other questions that will surely follow require well-designed studies that incorporate molecular, genetic, metabolic and nutritional factors.

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REVIEW

Sedation in gastrointestinal endoscopy: Current issues

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Abstract

Diagnostic and therapeutic endoscopy can successfully be performed by applying moderate (conscious) sedation. Moderate sedation, using midazolam and an opioid, is the standard method of sedation, although propofol is increasingly being used in many countries because the satisfaction of endoscopists with propofol sedation is greater compared with their satisfaction with conventional sedation. Moreover, the use of propofol is currently preferred for the endoscopic sedation of patients with advanced liver disease due to its short biologic half-life and, consequently, its low risk of inducing hepatic encephalopathy. In the future, propofol could become the preferred sedation agent, especially for routine colonoscopy. Midazolam is the benzodiazepine of choice because of its shorter duration of action and better pharmacokinetic profile compared with diazepam. Among opioids, pethidine and fentanyl are the most popular. A number of other substances have been tested in several clinical trials with promising results. Among them, newer opioids, such as remifentanil, enable a faster recovery. The controversy regarding the administration of sedation by an endoscopist or an experienced nurse, as well as the optimal staffing of endoscopy units, continues to be a matter of discussion. Safe sedation in special clinical circumstances, such as in the cases of obese, pregnant, and elderly individuals, as well as patients with chronic lung, renal or liver disease, requires modification of the dose of the drugs used for sedation. In the great majority of patients, sedation under the supervision of a properly trained endoscopist remains the standard practice worldwide. In this review, an overview of the current knowledge concerning sedation during digestive endoscopy will be provided based on the data in the current literature.

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Key words: Gastrointestinal endoscopy; Endoscopy; Sedation; Analgesia; Digestive system

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INTRODUCTION

Today, both the number and complexity of endoscopic procedures have increased considerably due to the wide availability and application of sedation, which facilitates successful endoscopic procedures because it can relieve patients' anxiety and discomfort, concurrently allowing them to experience a rapid recovery with the use of antidotes. Consequently, the willingness of patients to undergo endoscopy, irrespective of the severity of their situation, is increasing.

The best methods for analgesia and sedation during digestive endoscopy are still debated. Intravenous sedation can be administered by the endoscopist who concurrently performs the procedure while a qualified nurse monitors the patient's state of consciousness and vital signs. Providing an adequate regimen of sedation/analgesia may be considered a form of art, which influences,

for example, the quality of the examination and the patient's and physician's satisfaction with the sedation[1]. It must be argued that the optimal level of sedation differs according to the procedure being performed. Deep sedation or even general anaesthesia may be preferred for therapeutic procedures in which it is important for a patient to remain immobile. It is obvious that endoscopists commencing sedation/analgesia should be able to rescue patients whose level of sedation has become deeper than initially intended.

However, even today, many significant issues, such as the benefits, risks, and costs of sedation; the selection of the most suitable drug and combination of drugs for use; and the person responsible for the administration of sedation and monitoring of the patient, especially during time-consuming procedures such as colonoscopies and endoscopic retrograde cholangiopancreatography (ERCPs), remain unanswered[2].

The aim of this review is to provide the reader with an overview of the current knowledge concerning sedation during digestive endoscopy (drugs currently used and drugs under investigation, sedation during upper and lower gastrointestinal (GI) endoscopy and ERCP, and endoscopy in special situations) based on the data available in the current literature.

SEDATION PRACTICES

Sedation practices vary in different countries depending on health system regulations and local circumstances. On the other hand, differences in the setting in which the practice of gastroenterology and endoscopy takes place, e.g., at university hospitals versus community hospitals or private endoscopy units, as well as other systematic practice differences, could influence the attitude of endoscopists concerning sedation practices.

Data concerning the incidence of sedation application in routine practice are rather limited. Among the members of the Canadian Association of Gastroenterology, more than 90% use sedation during colonoscopy[3]. The use of sedation has become a standard practice during GI endoscopy in Italy[4]. Among the members of the Hellenic Society of Gastroenterology, 64% use sedation regularly in cases of upper GI endoscopy, 78% use sedation in colonoscopies, and 100% use sedation during ERCP and endoscopic ultrasound (EUS)[5]. In the United States, more than 98% of colonoscopies are performed with intravenous sedation[6]. In Switzerland, the use of sedation in GI endoscopy has markedly increased, and the use of electronic monitoring has become a standard practice. In this country and during 2003, sedation was used in 78% of upper and lower GI endoscopic procedures, compared with 60% in 1990. In Germany, the majority of esophagogastroduodenoscopy (EGDs; 74%) and colonoscopies (87%) are carried out under sedation[7]. In Spain, sedation is used in 20% of EGDs and 20% of colonoscopies, while ERCP is almost always performed under sedation[8].

With regard to the most common sedation regimen used in different countries, it was reported that most Canadian endoscopists use a combination of midazolam and fentanyl for colonoscopy, while propofol, either alone or in combination with other drugs, is used in a small proportion of patients[3]. Interestingly, a significantly higher proportion of adult gastroenterologists who routinely used propofol are highly satisfied compared with those using other drugs. According to a recent survey among the members of the Italian Society of Digestive Endoscopy, the most commonly employed sedation patterns are benzodiazepines for upper GI endoscopy (50.8%), benzodiazepines plus opioids for colonoscopy (39.5%) and enteroscopy (35.3%), and propofol for ERCP (42.3%) and EUS (35.6%). Concerning the use of propofol, 66% of endoscopists stated that the drug was administered exclusively by anaesthesiologists[4]. In Greece, 62.1% of the endoscopists use synergistic sedation (benzodiazepines plus opioids), 35.3% use benzodiazepines alone and 33.8% use propofol-based sedation in selected cases. Propofol administration is directed by an anaesthesiologist in most cases[5]. In the United States, more than 75% of endoscopists use a benzodiazepine plus narcotic combination, with the combination of midazolam and fentanyl being the most common[6]. In Switzerland, midazolam is the most commonly used medication. The drug is administered by the endoscopy nurse *via* an intravenous cannula. A significant percentage of endoscopists (43%) also use propofol regularly, mainly in a hospital setting. Endoscopists reporting the use of propofol without the assistance of an anaesthesiologist had performed a total of 82 620 procedures. The morbidity in this group of patients was 0.19%, with no cases of mortality[9]. The doses of midazolam and fentanyl used by the Canadian endoscopists are similar to those recommended in the United States (< 6 mg of midazolam and < 200 μg of fentanyl) and in the United Kingdom (< 5 mg of midazolam and < 100 μg of fentanyl)[10]. In Spain, the most common drugs were midazolam for gastroscopy and midazolam and pethidine for colonoscopy and ERCP, while propofol is most frequently used by anaesthesiologists[8]. In Germany, the most frequently used agents for sedation are midazolam (82%) and propofol (74%), and the most common sedation regimens used are propofol plus benzodiazepines (38%) and benzodiazepines plus an opioid (35%)[7].

In Italy, pulse oximetry is the most common system for patient monitoring during endoscopy, while supplemental O_2 is routinely administered by 39.3% of endoscopists[4]. In Greece, pulse oximetry is used in 96% of endoscopic procedures[5]. Major endoscopy societies, including those of Canada and the United States, recommend the use of pulse oximetry, continuous electrocardiogram and blood pressure, and heart rate monitoring in patients receiving propofol[11,12]. In Switzerland, pulse oximetry monitoring is currently used in more than 95% of examinations, compared with 2.5% of examinations in 1990[9]. In Germany, all patients are routinely moni-

Table 1 Percentage of sedation use in different countries

Country	Sedation	Propofol use	Benzodia-zepines alone use	Benzodiaze-pines plus opioids use	No. of nurses present during endoscopy	Pulse oximetry use	Supplemental oxygen use
Canada	90%	12%		always	1		
Italy		42.30% for ERCP (by anaesthesiologists)	50.80%	39.50%		100%	By 39.3% of endoscopists
Greece	EGD: 64%; Colonoscopy: 78%; ERCP: 100%; EUS: 100%	33.80% (in selected cases and only by anaesthesiologists)	35.30%	62.10%		96%	
United States	98%	25.70%		74.30%		98.60%	By 72.7% of endoscopists (in all EGDs)
Switzerland	78%	43% (regular use with or without the help of an anaesthesiologist)	Midazolam for the majority of endoscopies		1	95%	
Spain	EGD: 20%; Colonoscopy: 20%; ERCP: 100%; EGD: 74%	Only by anaesthesiologists	Only for EFD	Only for colonoscopies	1	77%	
Germany	Colonoscopy: 87%	74%	82%	35%		97%	34%

ERCP: Endoscopic retrograde cholangiopancreatography; EGD: Esophagogastroduodenoscopy; EUS: Endoscopic ultrasound; EFD: Energy flux density.

tored by pulse oximetry, while automated blood pressure monitoring and/or electrocardiography are applied in 29 and 13% of cases, respectively. Supplemental oxygen is routinely administered to 34% of patients[7].

Data concerning endoscopy practices in developing nations are scarce. In a study comparing endoscopy practices between endoscopists practising in 46 developed and developing countries, no significant differences in the use of a benzodiazepine and opioid combination, propofol alone, or unsedated endoscopy were found. Sedation is used for most endoscopic procedures, leading to the conclusion that sedation practices do not significantly differ between developing and developed countries[13].

Table 1 provides a summary of sedation practices in some parts of the world.

DRUGS CURRENTLY USED FOR SEDATION IN GI ENDOSCOPY

Various types of sedation and analgesia techniques are used during GI endoscopy procedures. Currently, there is no standard sedation regimen, and even within individual institutions, the choice of sedation may depend on endoscopist preference and the procedure being performed. Benzodiazepines, such as alprazolam, bromazepam, brotizolam, clotiazepam, diazepam, etizolam, flunitrazepam, lorazepam, midazolam, oxazepam and triazolam, are among the most frequently prescribed drugs. These drugs act as anxiolytics, sedatives, hypnotics, amnesics, antiepileptics and muscle relaxants. Among them, midazolam is an important drug and is widely used in everyday endoscopy work. It is now considered to be the benzodiazepine of choice, as it has a shorter duration of action and a better pharmacokinetic profile than diazepam. Other drugs used for sedation include opioids (pethidine and fentanyl), propofol, ketamine and droperidol. Adequate knowledge of the pharmacokinetic properties of these agents is crucial when commencing sedation (Table 2)[14].

The special characteristics of the currently available drugs for digestive endoscopy, as well as the drugs under investigation, are shown in Table 3 and are subsequently summarised in the following sections.

Midazolam

Midazolam is most likely the most widely used drug for sedation in everyday endoscopic work. The action of midazolam is due to the potentiation of the neural inhibition mediated by gamma-aminobutyric acid. In addition to its action on the central nervous system, midazolam exhibits a dose-dependent ventilatory depressant effect and causes a reduction in arterial blood pressure and an increase in heart rate. Midazolam is metabolised by cytochrome P450 enzymes and glucuronide conjugation. CYP3A4 is important in the biotransformation of midazolam.

The duration of action of midazolam is dependent on the duration of its administration. It also has synergistic interactions with other hypnotics and opioids. Various factors, including age, compromised renal function, and liver dysfunction, affect the pharmacokinetics of the drug[15].

Clinical studies: A large number of prospective, randomised, placebo-controlled and non-controlled trials have been published in recent years concerning the efficacy and safety of midazolam alone or in combination with analgesics. The most recent of these trials are mentioned below.

In a study conducted to evaluate the prevalence of hypoxia related to midazolam sedation during upper GI endoscopy, 180 patients referred for selective endoscopy were randomised to either midazolam sedation or pla-

Table 2 Characteristics of the pharmacological agents used to achieve a moderate level of sedation in gastrointestinal endoscopy (*i.v.* administration)[1]

Agent	Chemical structure	Molecular weight (g/moL)	Onset of action (min)	Duration of action	Elimination half-life	Metabolism/excretion
Midazolam	$C_{18}H_{13}ClFN_3$	325.78	1.0-2.5	2-6 h	1.8-6.4 h	Hepatic and intestinal; excreted in urine
Propofol	$C_{12}H_{18}O$	178.27	< 1	3-10 min	Triphasic: 2.2 min, 20 min, 8 h	Hepatic; excreted in urine
Fentanyl	$C_{22}H_{28}N_2O$	336.471	⩽ 1.5	1-2 h	2-7 h	Hepatic; excreted in urine
Meperidine	$C_{15}H_{21}NO_2$	247.33	5	2-4 h	2-7 h	Hepatic; excreted in urine

[1]Modified from Manolaraki *et al*[14].

Table 3 Currently used drugs for sedation and drugs under investigation

Drugs currently used for sedation	Drugs and other practices under investigation
Midazolam	Nitrous oxide gas (N_2O)
Fentanyl	Remimazolam
Propofol	Fospropofol
	Dexmedetomidine
	Alfentanyl
	Remifentanil
	Music

cebo. The results revealed that no patients developed any serious episodes of hypoxia and that the incidence of mild hypoxia was not significantly different between the two groups. There was no significant difference in arterial oxygen saturation as recorded by the endoscopist staff[16].

In another study, haemodynamic responses during gastroscopy in healthy subjects were studied in two groups: midazolam alone *vs* midazolam in combination with meperidine. It was found that blood pressure and oxygen saturation significantly decreased with sedation in both groups during endoscopy, but no significant differences were found between the two groups. Heart rate increased significantly, whereas systolic arterial pressure, diastolic arterial pressure and O_2 saturation (SpO_2) decreased significantly, with both regimes. Patient compliance was significantly better with combined sedation[17].

Midazolam has been tested in combination with a variety of other drugs. In one study, the efficacy and safety of midazolam in combination were tested in 74 patients. The midazolam group received only midazolam, and the midazolam/meperidine group received midazolam plus meperidine. The results showed that there was no significant difference between the two groups with regard to the recovery time, procedure time and mean visual analogue scale scores[18].

In a randomised trial comparing the efficacy and recovery time of two sedation regimens consisting of midazolam in combination with either meperidine or fentanyl in patients submitted for colonoscopy, it was found that the use of fentanyl in combination with low-dose midazolam resulted in a significantly faster recovery from sedation compared with meperidine, without any apparent loss of analgesic effect[19].

The synergistic sedation with low-dose midazolam plus propofol *vs* the standard regimen of midazolam and pethidine for conscious sedation in colonoscopy was investigated in a group of patients that included a large number of elderly patients with comorbidities. The synergistic sedation with low-dose midazolam plus propofol was superior to a standard combination of midazolam and the opioid pethidine for colonoscopy in terms of patient comfort and recovery time[20].

In a prospective, randomised study, the standard regime of midazolam and pethidine *vs* a propofol-fentanyl mixture was tested. It was found that patient-controlled sedation/analgesia with propofol and fentanyl was a safe and effective combination, resulting in a high level of satisfaction for both the patient undergoing upper GI tract endoscopic ultrasonography and the endoscopist[21].

Barriga *et al*[22] evaluated the adequacy of conscious sedation during upper endoscopy using midazolam alone compared with midazolam plus fentanyl. Although, from the endoscopist's perspective, patients in the combination group had better tolerance, no significant differences were found in the patient assessments. These results suggest that an adequate level of sedation can be obtained safely by either midazolam or midazolam plus fentanyl.

Midazolam was also tested as an orally administered premedication in patients undergoing upper GI endoscopy. In a double-blind, placebo-controlled, randomised trial, 130 patients were randomised to receive either 7.5 mg of midazolam orally or a placebo as the premedication. The results showed that the median anxiety score during the procedure in the midazolam group was significantly lower than that in the control group. Moreover, a significantly greater number of patients in the midazolam group graded overall tolerance as "excellent or good" and reported partial or complete amnesia in greater degree compared with the control group. Finally, patients in the midazolam group were more willing to repeat the procedure if necessary. However, the median recovery time was significantly longer in the midazolam group than in the control group. No significant differences in the satisfaction score and haemodynamic changes between the two groups were observed[23].

In conclusion, midazolam must be considered as an excellent drug for achieving safe and effective sedation during upper and lower GI endoscopy, whether used alone or in combination with analgesics.

Propofol

The sedative-hypnotic drug propofol (2,6-diisopropylphenol) is a phenolic derivative with satisfactory sedative,

hypnotic, antiemetic and amnesic properties. Additionally, propofol the advantage of a rapid onset of action and a short recovery profile. The depth of sedation increases in a dose-dependent manner. Propofol is highly lipophilic and, therefore, can rapidly cross the blood-brain barrier, resulting in an early onset of action. Consequently, emergence from sedation is also quite rapid because of its fast redistribution into peripheral tissues. Sedation with propofol can be achieved both by bolus administration and continuous infusion. The drug can cause unconsciousness within 30 s. As an additional advantage, regardless of the length of the sedation period, recovery from propofol will occur within 10-20 min after discontinuation. Propofol also has an excellent amnesic effect and short half-life (4 min *vs* 30 min for midazolam). Currently, there is no dispute regarding propofol's superiority over benzodiazepines (with or without opioids) in terms of the abovementioned physiological effects. However, it must be strongly emphasised that titrating propofol to achieve conscious sedation without inducing general anaesthesia requires significant clinical expertise.

This drug is being increasingly used for sedation during painful diagnostic and therapeutic procedures because it increases the quality of upper GI endoscopy by increasing patients' acceptance of the procedure and improving the diagnostic accuracy of endoscopy[24].

With regard to side effects, propofol is generally associated with good haemodynamic stability, although it can induce a dose-dependent decrease in blood pressure and heart rate. Transient decreases in blood pressure are more prominent during bolus administration. Thus, slow initial infusions are recommended in most patients. Moreover, strict aseptic technique must be used during the handling of the product to prevent accidental extrinsic microbial contamination. There are also some other disadvantages of propofol, including the lack of a pharmacological antagonist. The combination of propofol and midazolam has synergistic effects and may have advantages over the use of propofol as a single agent[20]. Thus, a combined sedation regimen with a benzodiazepine retains the possibility for partial pharmacological reversibility using flumazenil.

However, data from a recent meta-analysis suggest that propofol sedation is not associated with an increased risk of complications. In fact, propofol sedation for colonoscopy was associated with lower complication rates than sedation with traditional agents[25]. Several prospective studies confirmed that lower doses were needed for combined sedation with midazolam/propofol compared with propofol alone during diagnostic or therapeutic endoscopy[26,27].

A large number of clinical trials and meta-analyses of these trials have been published, the main results of which are discussed below.

Clinical studies of propofol in upper GI tract endoscopy: A number of studies revealed that propofol offers significant advantages over benzodiazepines and opioids for sedation during endoscopic procedures. Other prospective studies indicated that propofol was safer and more effective than midazolam and meperidine for reaching and maintaining an adequate level of sedation during endoscopy, resulting in better titration of the level of sedation and a shorter recovery time[28].

A prospective study evaluated the safety and efficacy of nurse-administered, low-dose propofol sedation in 8431 adults submitted to upper GI endoscopy. Propofol was administered by bolus injection at a dose of 40 mg for patients < 70 years old, 30 mg for patients 70-89 years old, and 20 mg for patients 90 years old or older. Only 0.26% of the patients required a transient supplemental oxygen supply, and full recovery occurred in 99.9% of patients 60 min after the procedure. However, men and younger patients required significantly higher doses of propofol than women and older patients. A total of 99% of patients were willing to repeat the same procedure. This study showed that the use of only a low dose of nurse-administered propofol sedation is safe and effective for diagnostic esophagogastro-duodenoscopy[29].

Levitzky *et al*[30] showed that balanced propofol sedation targeted to induce moderate sedation in patients undergoing upper GI endoscopy results in better patient satisfaction and a shorter recovery time than standard sedation alone.

Propofol in lower GI tract endoscopy: The optimal regimen of propofol for colonoscopy sedation is still controversial. Both propofol alone and propofol in combination with opiates (meperidine) or benzodiazepines (midazolam) are frequently used during colonoscopy to achieve moderate levels of sedation. Hsieh *et al*[31] noticed that for sedated colonoscopy, propofol in combination with meperidine is better than propofol alone for improving patients' tolerance and recovery.

The combination of 1.0-2.0 mg of midazolam with either 50-100 mg of fentanyl or *i.v.* propofol of 0.5-2.5 mg/kg allowed patients to undergo colonoscopy under comparable sedative and analgesic conditions. The combination with fentanyl had a significantly smaller effect on pulse rate and blood pressure, while the combination with propofol produced more favourable results, especially in terms of superior amnestic effects[32].

In a study of 300 adults undergoing colonoscopy, the use of fentanyl in combination with low-dose midazolam was found to result in a faster recovery from sedation compared with meperidine without decreasing the analgesic effect[19].

Recently, patient-controlled sedation with propofol has been advocated as a method for dealing with the narrow therapeutic window for moderate sedation. In a relevant study, 50 patients undergoing elective colonoscopy were randomised to receive midazolam/fentanyl or propofol/remifentanil administered *via* patient-controlled sedation. Patients in the propofol/remifentanil group were sedated and recovered significantly more rapidly than patients in the midazolam/fentanyl group[33].

Propofor in therapeutic GI endoscopic procedures: In a prospective, randomised, single-blinded study of 222 patients, Lee *et al*[34] compared the safety and efficacy of balanced propofol sedation with conventional sedation (midazolam and meperidine) in patients undergoing therapeutic endoscopic procedures. They found no significant differences between the balanced propofol sedation and conventional groups with regard to the rates of cardiopulmonary complications and transient interruption of procedures, although balanced propofol sedation provided a significantly higher level of endoscopist satisfaction and better patient cooperation.

Meta-analyses of the use of propofol for sedation: To date, two meta-analyses have been published concerning the safety and efficacy of propofol for GI endoscopy.

The first meta-analysis, published in 2005, included 12 original studies with 1161 patients. Of these patients, 634 received propofol and 527 received midazolam, meperidine, and/or fentanyl. Most of the studies were randomised trials of moderate quality. It was found that the pooled odds ratio for developing hypoxia or hypotension for all of the procedures combined was 0.74 in patients using propofol. The pooled odds ratios were 0.85 for upper GI endoscopy, 0.4 for colonoscopy, and 1.07 for ERCP/EUS. Compared with traditional agents, it was noted that sedation with propofol during colonoscopy appears to result in a lower incidence of cardiopulmonary complications, although the risk of complications associated with upper GI endoscopy seems to be similar[25].

The second meta-analysis included 20 studies on the use of propofol for colonoscopy[35]. The analysis showed that recovery and discharge times were shorter with the use of propofol. There was also higher patient satisfaction with the use of propofol, although no significant differences in the procedure time, cecal intubation rate and number of complications were noticed. Finally, no difference in pain control with non-patient-controlled sedation with propofol compared with the traditional agents was noted. The only disadvantage was that pain control with propofol was inferior compared with the use of traditional agents. The general conclusion from these meta-analyses is that propofol for sedation during colonoscopy results in a faster recovery and discharge time, as well as increased patient satisfaction and acceptance rates of side effects.

In conclusion, propofol provides a faster onset of action and deeper sedation compared with standard doses of benzodiazepines and narcotics. More rapid cognitive and functional recovery should be expected when propofol is used as a single agent compared with benzodiazepines. The drug appears to have more advantages when used for prolonged and therapeutic endoscopic procedures, including ERCP and EUS. Although it can be safely and effectively used by a physician-supervised nurse, patients must be under continuous care, consistent with the care required for patients undergoing deep sedation. Personnel in the endoscopy room should be able to rescue the patient from severe respiratory depression.

Fentanyl

Fentanyl, a μ-opioid receptor agonist, is a synthetic narcotic analgesic characterised by a rapid onset and short duration of action. The action of the drug is related to its agonism of the opioid receptors. It is 100 times more potent than morphine, with 100 μg equivalent to 10 mg of morphine and 75 mg of meperidine (pethidine) in terms of analgesic activity. Its strong potency is largely due to its high lipophilicity, which also explains the rapid penetration of the drug into the central nervous system. Fentanyl binds μ-opioid G-protein-coupled receptors, which inhibits the release of pain neurotransmitters by decreasing intracellular Ca^{2+} levels. It has been used in combination with midazolam, mainly in patients undergoing lower GI endoscopy.

In a trial comparing meperidine with fentanyl, the authors noted that the total procedure time was shorter for those receiving fentanyl than for those receiving meperidine. Based on post-procedure pain scores, examinations performed using meperidine were less painful compared with those performed with fentanyl[36].

A meta-analysis compared the efficacy, safety, and efficiency of agents used for moderate sedation in upper GI endoscopy or colonoscopy in 36 studies involving a total of 3918 patients. Sedation improved patient satisfaction and the willingness of patients to repeat upper GI endoscopy compared with these measures in patients who received no sedation. Midazolam provided superior patient satisfaction and resulted in a less frequent memory of the upper GI endoscopy procedure compared with diazepam. Adverse events and patient/physician assessments were not different between midazolam (with or without narcotics) and propofol. The procedure time was similar, but sedation and recovery times were shorter with propofol than midazolam-based regimens. The results confirmed that moderate sedation provides a higher level of physician and patient satisfaction and a lower risk of serious adverse events compared with other currently available agents. Midazolam-based regimens have longer sedation and recovery times than propofol[37].

DRUGS UNDER INVESTIGATION FOR GI ENDOSCOPY

Various other drugs are also under investigation for GI endoscopy. Among them, prodrug formulations of propofol have been developed to overcome the disadvantages of the lipid-based formulations. So far, the results of the relevant studies appear promising. The most important clinical data are presented below.

Nitrous oxide gas

Nitrous oxide gas (N_2O) (molecular mass 44.013) has been proposed as an alternative to *i.v.* analgesia in patients undergoing lower GI endoscopy. N_2O/O_2 mixtures have a satisfactory analgesic effect and short half-lives, thus providing an alternative method of sedation for colonoscopy procedures.

A systematic review of 11 randomised studies including 623 patients was published in 2010[38]. In these studies, N_2O was compared with a lack of sedation in patients undergoing either flexible sigmoidoscopy or colonoscopy. The results revealed that patient-reported pain was similar for N_2O when undergoing flexible sigmoidoscopy *vs* no sedation and when undergoing colonoscopy *vs i.v.* sedation. No differences in duration, procedure difficulty or complications were identified. N_2O was associated with a more rapid recovery than *i.v.* sedation. This systematic review supports the assumption that N_2O provides comparable analgesia to *i.v.* sedation in patients undergoing colonoscopy. Rapid recovery enables quicker patient discharge and removes the need for a patient to be chaperoned.

In a more recent Cochrane review, 257 patients were randomised to receive a N_2O/O_2 mixture (7 studies), while 225 patients received some form of sedation with or without additional analgesia (6 studies) and 65 patients received placebo (3 studies). Four studies showed that N_2O/O_2 reduced pain/discomfort compared with conventional methods, whereas one study showed that sedation was better and another study showed that N_2O/O_2 was better. Six studies showed that N_2O/O_2 groups had a quicker recovery time and shorter length of hospital stay, whereas one study showed no difference between the two groups. Two studies showed that N_2O/O_2 was safer, whereas one showed that sedation was safer. The conclusion was that N_2O is as efficient as and safer than other pain relief methods used during colonoscopy[39].

A randomised clinical trial compared the efficacy of Entonox (50% N_2O and 50% O_2) with midazolam-fentanyl sedation in 131 patients undergoing elective colonoscopy. Sixty-five patients received Entonox, and 66 patients received midazolam-fentanyl. Patients receiving Entonox had a shorter time to discharge. They also reported significantly less pain and better recovery of psychomotor function immediately after the procedure and at discharge. Patients who received Entonox also reported a higher level of satisfaction. Again, this study concluded that Entonox provides better pain relief and faster recovery than midazolam-fentanyl in patients undergoing elective colonoscopy[40].

A double-blind, randomised, placebo-controlled trial showed that N_2O inhaled intermittently is not an effective substitution for *i.v.* on-demand sedation in the setting of colonoscopy without sedation. In this study, patients inhaled N_2O or placebo on demand. The median patient-reported pain level was 2 in both the N_2O and control groups. Additional sedatives and analgesics were given equally often and at similar doses in both groups. No side effects related to the administration of N_2O were noted[41].

In conclusion, the available data suggest that N_2O is an effective analgesic and sedative agent that must be further investigated in larger studies.

Remimazolam

Remimazolam ($C_{21}H_{19}B_rN_4O_2$; molecular weight 439.304) is a short-acting GABA(A) receptor agonist that exhibits organ-independent metabolism, which was developed as an *i.v.* sedative agent for use in day-case procedures and the induction and maintenance of anaesthesia.

Preclinical studies in animals demonstrated that remimazolam caused a more rapid onset and a shorter duration of action compared with midazolam.

In a phase Ⅱ a clinical trial evaluating remimazolam as a procedural sedative for upper GI endoscopy, the time to recovery from sedation was shorter and more consistent with remimazolam compared with midazolam. Because of its organ-independent metabolism and rapid and predictable onset and recovery profile, remimazolam appears to have potential advantages over other currently available short-acting benzodiazepines. However, its respiratory depressant effect has been reported in numerous studies[42].

Fospropofol

Various prodrug formulations of propofol have been developed to overcome the disadvantages of the lipid-based formulations, the complications of lipid infusion, and the risk of fluctuations in propofol plasma levels due to bolus injection.

Fospropofol ($C_{13}H_{19}Na_2O_5P$, molecular weight: 332.24) is a water-soluble prodrug of propofol, metabolised *in vivo* to produce liberated propofol (producing the sedative effect), phosphate and formaldehyde. After *i.v.* injection, propofol is released from fospropofol by tissue alkaline phosphatases with a pattern of plasma concentrations, resulting in lower peak concentrations and a more gradual decline in drug concentrations compared with standard propofol administration protocols. As a result, the drug has pharmacokinetic and pharmacodynamic properties that differ from those of propofol emulsion. The time of the peak sedative effect after a bolus injection fluctuates between 3 and 7.5 min, compared with 1 min 36 s for propofol[43]. Fospropofol was generally well tolerated in clinical trials. Adverse events are mostly of mild-to-moderate severity and are transient and self-limiting[44].

The 6.5-mg/kg dose of fospropofol provides the ideal balance of efficacy and safety for patients undergoing colonoscopy. In a double-blind trial evaluating 127 patients who received fospropofol (2, 5, 6.5 or 8 mg/kg) or midazolam 0.02 mg/kg following pre-treatment with fentanyl, fospropofol yielded a significant dose-dependent increase in sedation success from 24% (2 mg/kg), 35% (5 mg/kg) and 69% (6.5 mg/kg) to 96% (8 mg/kg; $P < 0.001$)[45].

The same group of authors evaluated the efficacy and safety of *i.v.* fospropofol administration in patients undergoing colonoscopy for moderate sedation. Patients were randomised to receive fospropofol 2 mg/kg, fospropofol 6.5 mg/kg, or midazolam 0.02 mg/kg after pretreatment with *i.v.* fentanyl 50 μg. The results showed that sedation success was higher in the fospropofol 6.5 mg/kg group compared with the 2 mg/kg group (87% *vs* 26%; $P < 0.001$) and was 69% in the midazolam group.

Most adverse events were mild to moderate in intensity, the most common being paraesthesias (68% *vs* 60%) and pruritus (16% *vs* 26%) in the fospropofol 6.5 and 2 mg/kg groups, respectively. Fospropofol 6.5 mg/kg was associated with higher rates of sedation success, memory retention, and physician satisfaction than fospropofol 2 mg/kg[46].

Together, these results suggest that fospropofol is a promising drug requiring further investigation.

Dexmedetomidine

Dexmedetomidine ($C_{13}H_{16}N_2$, molecular mass: 200.28), a pharmacologically active dextroisomer of medetomidine, is a selective α(2)-adrenergic receptor agonist. It is indicated for the sedation of mechanically ventilated adult patients in an intensive care setting and in non-intubated adult patients prior to and/or during surgical and other procedures[47]. The drug should be administered *i.v.* only by experienced individuals, and the patient must be continuously monitored. Additionally, the dose must be adjusted in patients with liver and renal failure, as well as in elderly patients.

Dexmedetomidine can be safely used as a sedoanalgesic agent in colonoscopies because it provides efficient haemodynamic stability, higher satisfaction scores and lower Numeric Rating Scale scores. A study comparing dexmedetomidine (1 μg/kg and as a continuous infusion dose of 0.5 μg/kg per hour) with midazolam (0.05 mg/kg) plus fentanyl citrate (1 μg/kg) with regard to perioperative haemodynamics, sedation, pain, satisfaction and recovery scores during colonoscopy showed that, although statistically significant differences in mean arterial pressure were not detected between the two groups, heart rates were higher and SpO_2 scores were lower in dexmedetomidine group. When the groups were compared using the Ramsay sedation scale, the scores of group I at the 10th and 15th minute were significantly lower than those of group II[48].

In a recent study, Takimoto *et al*[49] showed that sedation with dexmedetomidine is a safe and effective practice in patients with gastric tumours undergoing endoscopic mucosal resection. In their study, 90 patients with gastric tumours were sedated with either dexmedetomidine [*i.v.* infusion of 3.0 μg/kg per hour over 5 min, followed by continuous infusion at 0.4 μg/kg per hour (n = 30), propofol (n = 30), or midazolam (n = 30)]. In all groups, 1 mg of dexmedetomidine was added *i.v.* as needed. The results showed that none of the dexmedetomidine-sedated patients exhibited a significant reduction in oxygen saturation level. Fewer patients in the dexmedetomidine group showed body movement during endoscopy compared with the other groups. The rate of effective sedation was significantly higher in the dexmedetomidine group compared with the midazolam and propofol groups. The mean duration of endoscopic submucosal dissection in the dexmedetomidine group was significantly shorter than that in the other two groups. However, dexmedetomidine alone is most likely not as effective as propofol combined with fentanyl for providing conscious sedation during ERCP, exhibiting concurrently greater haemodynamic instability and prolonged recovery[50].

Alfentanyl

Alfentanyl ($C_{21}H_{32}N_6O_3$, molecular weight: 452.98) is a narcotic analgesic with a rapid onset of action, a very short duration of action, and a potency of approximately one-third that of fentanyl. Recently, it was shown that patient-controlled analgesia pumps and sedation with alfentanyl and fentanyl for colonoscopy are safe, feasible, and acceptable to most patients, although a shorter sedation time makes alfentanyl more attractive, as it reduces the postprocedural workload[51].

Remifentanil

Remifentanil ($C_{20}H_{28}N_2O_5$, molecular weight: 376.447) is a μ-opioid receptor agonist that has important neuroanaesthesia characteristics. It has been used in a small number of clinical trials in patients undergoing GI endoscopic procedures. There are many reports of the use of remifentanil in different settings, including GI endoscopy, with or without background infusion, and the quality of analgesia and patient satisfaction seem to be the same as with standard sedation/analgesia. It seems that remifentanil patient-controlled analgesia is safe and effective for inducing sedoanalgesia during colonoscopy.

In a randomised, double-blind clinical trial, 60 patients undergoing colonoscopy were randomly assigned to either the remifentanil or meperidine group. All of the patients received premedication with midazolam 0.03 mg/kg *i.v.* In the remifentanil group, a bolus dose of remifentanil was given, and a patient-controlled sedation/analgesia pump was set to inject further bolus doses, while patients in the meperidine group received a bolus of meperidine and a sham, patient-controlled sedation analgesia pump. The degree of pain, level of satisfaction with sedoanalgesia of patients and gastroenterologists, and degree of difficulty experienced by the endoscopist, as well as the discharge time and duration of colonoscopy, were not different between the two groups[52].

In another study, the safety and efficacy of remifentanil during colonoscopy compared with the standard combination of midazolam and pethidine were tested in 116 patients who received either midazolam and pethidine or remifentanil only. Recovery was found to be faster in the remifentanil group. There was also a significant difference with regard to the time of hospital discharge. In this study, remifentanil during colonoscopy provided sufficient pain relief with better haemodynamic stability, less respiratory depression, and significantly faster recovery and hospital discharge times than moderate sedation with midazolam and pethidine[53]. However, further studies are needed to confirm these interesting results.

Music

Among methods reported to minimise patient discomfort during GI endoscopy (especially colonoscopy), mu-

sic has been utilised as an important therapeutic tool for effectively relieving stress and inducing analgesia. During recent years, several papers investigating the efficacy of music on patients' stress and pain relief were published. The most important of these publications are discussed below.

Clinical trials concerning the role of music in sedation: More recent clinical trials not included in the abovementioned meta-analyses have revealed rather conflicting results.

In a single-blind, randomised, controlled trial, the authors showed that music significantly reduces discomfort and, consequently, should be routinely provided to patients undergoing colonoscopy. In this study, 109 patients were randomised to receive music-delivering or non-sound-emitting headphones before and during endoscopy. The results revealed that the mean pain score was significantly lower in the music group compared with the control group, while overall satisfaction and willingness to repeat the procedure were significantly improved and the difficulty perceived by physicians was significantly reduced. Interestingly, the total amount of midazolam and pethidine was significantly lower in the music group compared with the control group[54].

Music in the endoscopy room was also found to reduce the anxiety levels in patients undergoing endoscopic procedures. In a controlled trial of 180 patients, the effect of age and type of endoscopic procedure on anxiety levels upon arrival in the unit and immediately before the endoscopy procedure after listening to music or no music (control group) for the same period was investigated. Although anxiety levels were not influenced by age or procedure, it was found that listening to music resulted in a significant reduction in anxiety scores, which was maintained for all age groups, irrespective of the type of endoscopic procedure performed. The authors suggest that providing music in the endoscopy unit is a simple strategy that can improve the well-being of patients[55].

Another study, which was specifically designed to investigate whether listening to music reduced the pain experienced by patients during sigmoidoscopy without sedation or analgesia, concluded that listening to music did not reduce pain intensity. In this study, it was found that the mean pain intensity in the music group was not different from that in the control group, and the proportion of patients with at least moderate pain during sigmoidoscopy did not differ between the two groups[56].

Meta-analyses concerning the role of music in sedation: Three meta-analyses regarding the role of music in sedation have been published to date. At least two of them suggested that music can effectively relieve stress and improve the level of analgesia during GI endoscopy. In the first of these meta-analyses, the authors included six randomised, controlled trials with a total of 641 patients. They found that in studies that did not use pharmacotherapy, patients receiving music therapy exhibited significantly lower anxiety levels compared with controls. Additionally, in studies in which pharmacotherapy was used, patients receiving music therapy exhibited significant reductions in analgesia requirements and an almost significant reduction in sedation requirements compared with controls. Furthermore, the procedure time was significantly reduced in the music therapy group compared with the control group. The authors' conclusion was that music therapy is an effective tool for stress relief and analgesia in patients undergoing GI endoscopic procedures[57].

In the second meta-analysis, which was published in 2008 and focused on colonoscopy, the authors included 8 studies with a total of 722 patients. In four studies, music was transmitted through headphones/earphones (as background music in three studies, and one study did not specify the media method). The results showed that the combined mean time taken for the colonoscopy procedure was shorter in the music group compared with the control group. There was weak evidence of benefit regarding the pain score, blood pressure, and mean recovery time in the music group compared with the control group. No harmful effects from listening to music were reported in any of the studies in this meta-analysis. The only disadvantage found in allowing patients to listen to music through headphones/earphones was the isolation of patients from the medical staff during the procedure. The authors concluded that "listening to music is effective in reducing procedure time and amount of sedation during colonoscopy and should be promoted"[58].

Finally, in the third meta-analysis, which was published in 2009 and included 8 studies with a total of 712 patients, it was found that patients' overall experience scores were significantly improved when they were allowed to listen to music. However, no significant differences were noted in patients' pain scores, mean doses of midazolam and meperidine, procedure time, and willingness to repeat the same procedure in the future, indicating that music improves only patients' overall experience with colonoscopy[59].

One possible explanation for the reduction in the doses used for sedation sedation is that patients in the music group are more relaxed and have less anxiety, resulting in a faster completion of the procedure and the use of less sedation[60]. The reduction in procedure time implies a reduction in the time during which patients feel anxious, frightened, and uncomfortable while undergoing the procedure and may be useful in enhancing the compliance rate. The avoidance of sedation may obviously result in a quicker patient discharge, less need for monitoring, and overall cost savings. Two other advantages of music are its inexpensiveness and ease of implementation[61].

In conclusion, it seems that listening to music, especially during colonoscopy, could reduce the procedure time, anxiety, and amount of sedation needed, without producing any harmful events. As a result, music should be promoted because of its beneficial effect and negligi-

ble cost. However, several aspects of this method, such as the choice of music and the mode of transmission, are worth further investigation.

SEDATION FOR GI ENDOSCOPY IN SPECIAL CLINICAL SITUATIONS

A large number of situations require special attention not only at the beginning of the endoscopic procedure but also during the procedure and recovery. The drugs that must be used, along with the precautions that must be followed, are analysed below.

Obesity

Obesity is a significant health problem that has assumed epidemic proportions. As a result, the number of obese patients requiring endoscopy is increasing. Morbid obesity can result in pulmonary hypertension, obstructive sleep apnoea, and restrictive lung disease. It is relatively unknown how safe the current practices of sedation for endoscopic procedures are in bariatric patients[62]. Therefore, special consideration should be given to these patients, and endoscopists need to be aware of challenges that may be present while performing endoscopic procedures in obese patients[63].

There are limited data on the use of sedation in obese patients. Studies published to date refer mainly to the use of sedation in obese subjects undergoing advanced endoscopic procedures or upper GI endoscopy before bariatric surgery.

In a study involving 69 subjects with morbid obesity submitted to upper GI endoscopy before bariatric surgery, the authors administered sedation with propofol at a mean dose of 380 ± 150 mg (range 80-900 mg). Two patients developed severe hypoxemia, which required bronchoscopic intratracheal O_2 insufflation. Thus, although upper GI endoscopy can be performed safely in obese patients, careful monitoring and anesthesiological support are required, especially in patients with concomitant diseases[64].

In a study investigating the safety of anaesthesia-assisted endoscopy using propofol-mediated sedation in subjects undergoing advanced endoscopic procedures, the authors found that an increased body mass index was associated with an increased frequency of airway manoeuvres and hypoxemia. A multivariate analysis revealed that body mass index was an independent predictor of the appearance of sedation-related complications. Interestingly, in obese individuals, there was no difference in the frequency of sedation-related complications in patients receiving propofol alone or in combination with other drugs. Propofol sedation can be safely used in obese patients undergoing advanced endoscopic procedures when administered by trained professionals, despite the increased frequency of sedation-related complications[65].

Finally, it was found that patients who undergo upper GI endoscopy with either anaesthesiologist- or surgeon-monitored sedation seem to tolerate the procedure equally well. However, significantly fewer patients in the anaesthesiologist-monitored sedation group complained of throat pain after the procedure and/or remembered gagging during the procedure, thus leading to the conclusion that anaesthesiologist-monitored sedation should be considered in patients undergoing preoperative upper endoscopy before bariatric surgery[66].

In conclusion, although quite safe, moderate sedation during endoscopy may pose some risks to obese patients. In particular, the presence of obstructive sleep apnoea may identify a subset of patients at higher risk of complications. Further studies are required in this field, as the number of subjects undergoing bariatric surgery is constantly increasing worldwide.

Chronic liver disease

Endoscopy, either diagnostic and/or therapeutic, is often necessary in patients with chronic liver disease, sometimes on an emergency basis. It is well established that liver disease may impair the metabolism of drugs usually administered for sedation. The evaluation of patients with chronic liver disease before endoscopy should include a full assessment of hepatic function, as well as a complete physical examination to exclude the possibility of the presence of hepatic encephalopathy. As a general rule, sedation should be used especially in patients undergoing ligation of acutely bleeding varices, although in some cases sedation is not necessary[67].

Liver dysfunction could reduce both the clearance of the drugs eliminated by hepatic metabolism or biliary excretion and plasma protein binding. Chronic liver disease is also associated with a reduction in drug-metabolising activities, such as the activity of the CYP450 enzymes. In patients with advanced cirrhosis, it is necessary to adjust the dose of those drugs eliminated by renal excretion[68].

Concerning the drugs used in the sedation of cirrhotic patients, most authors prefer to use propofol instead of benzodiazepines and opioids because of its short biological half-life and lower risk of provoking hepatic encephalopathy.

In a recently performed study, the authors compared sedation with combinations of propofol plus fentanyl and midazolam plus fentanyl in 210 cirrhotic patients undergoing upper GI endoscopy. The doses of midazolam and propofol were 0.05 and 0.25 mg/kg, respectively, while the dose of fentanyl was 50 μg *i.v.* in both groups. It was found that sedation with propofol was more effective and yielded a shorter recovery time than sedation with midazolam, indicating that it is a safe and effective regimen[69].

Another study reported that the use of propofol in patients with cirrhosis does not precipitate minimal or overt hepatic encephalopathy during upper GI endoscopy[70]. The results of this study were recently confirmed in a study showing that sedation with propofol in patients with compensated liver cirrhosis resulted in a shorter time to both recovery and discharge than midazolam, thus not exacerbating sub-clinical hepatic encephalopathy[71].

These results are in accordance with those described in a previous study that demonstrated that propofol sedation does not cause deterioration of minimal hepatic encephalopathy concurrently associated with improved recovery in patients with liver cirrhosis[72].

In conclusion, propofol represents a safe and effective sedation drug that could be used as an alternative to midazolam in patients with liver cirrhosis[73].

Pregnancy

Despite the fact that endoscopy is rarely required during pregnancy and is generally considered to be safe, endoscopists must be aware of the potential risks concerning both the mother and the foetus. Before endoscopy, the endoscopist must calculate the potential foetal risks, mainly due to sedation, and try to correct any possible maternal pathological situation, including hypoxia and hypotension.

During endoscopy, pregnant women should be carefully monitored by continuous electrocardiography, pulse oximetry, and intermittent blood pressure estimation[74].

Sedative drugs comprise a significant foetal risk during endoscopy in pregnant women because of the risk of hypoxia. Additionally, the exposure of pregnant women to radiation during ERCP represents another important risk that should be reduced as much as possible.

Currently, there is no evidence that endoscopy precipitates premature labour. If possible, endoscopic procedures must be performed without any sedation or, alternatively, by administering the lowest effective dose of sedative medication.

Regarding the types of drugs used for sedation, the available literature suggests that midazolam appears to be safe if used carefully[75].

ERCP is rarely necessary during pregnancy, although it cannot be avoided in pregnant women with recurrent biliary colic, abnormal liver function tests, and a dilated bile duct. A relevant study showed that ERCP can be safely performed during pregnancy, leading to successful treatment in almost all patients. Sedation is uneventful for all pregnant women and their foetuses. However, it must be recognised that pregnancy may be associated with a higher rate of post-ERCP pancreatitis compared with the general population[76]. The same conclusion was reached in another study, the authors of which noted that ERCP is a safe procedure during pregnancy, even if the placement of a biliary stent is necessary[77].

In conclusion, upper GI endoscopy, including therapeutic interventions such as the banding of oesophageal varices, seems to be relatively safe for the foetus, although it should be performed only when strongly indicated. Similarly, flexible sigmoidoscopy also appears to be safe for the foetus and, again, should only be performed when strongly indicated. Colonoscopy data suggest that this procedure should be performed only during the second trimester and only if there is a strong indication. Finally, ERCP seems to be relatively safe but should only be performed if there is a strong indication for its use. Sedation is rather safe in ERCP, and midazolam is the preferred pharmaceutical agent by most endoscopists. As a general rule, it may be suggested that endoscopy in pregnant women should always be performed in a hospital by an expert endoscopist and only when strongly indicated[78].

Sedation in celiac disease

It has been suggested that patients with celiac disease exhibit increased rates of neuropsychiatric disturbances and visceral hypersensitivity. In a retrospective cohort study, Lebwohl *et al*[79] noted that 26% of patients with celiac disease required higher amounts of both opioids and midazolam compared with age- and gender-matched controls, possibly due to increased visceral hypersensitivity, chronic opioid/anxiolytic use, and/or underlying neuropsychiatric illness.

Sedation in the elderly

Although GI endoscopy with sedation is increasingly performed in elderly patients, data on the outcomes and side effects of sedation are limited. Age-related pharmacokinetic changes and the presence of comorbidities and polypharmacy complicate drug therapy. Aging results in impairment in the function of multiple organs, including the liver, which may also affect drug metabolism and pharmacokinetics. In addition, older people often have to consume a variety of drugs, the bioavailability of which could be increased. Additionally, lipophilic drugs may have a prolonged half-life. Combined with reduced hepatic and renal clearance mechanisms, this prolonged half-life can prolong the recovery of elderly patients after sedation. In the elderly, hepatic drug clearance of some drugs can be reduced by up to 30%. Midazolam is indicated because there are no major differences in CYP3A4 activity between young and old people. Finally, renal excretion is decreased in most elderly individuals because of the presence of hypertension and coronary heart disease[80].

In the geriatric population, conscious sedation practices are modified by the administration of fewer agents at a slower rate and lower cumulative dose. Midazolam has been widely used in elderly patients[81]. Under certain circumstances, it seems that the benefits, in terms of tolerance of low-dose midazolam for upper GI endoscopic sedation, outweigh the risks in older people. Christe *et al*[82], in a randomised, double-blind, placebo-controlled study, administered either midazolam (30 μg/kg *i.v.*) or saline (placebo) to 65 geriatric inpatients undergoing upper GI endoscopy. The results revealed that midazolam increased the probability of good tolerance. Midazolam resulted in a 10-mmHg reduction in the mean arterial pressure without inducing clinically significant hypotension. Finally, midazolam was associated with a higher risk of hypoxemia after endoscopy, but not of confusion.

In a recent study, it was found that elderly patients submitted for endoscopy required lower mean propofol doses for sedation compared with patients aged < 70

years. No major complications and no difference in the number of minor complications were noted. A favourable safety profile for combined sedation with midazolam/propofol and a higher sensitivity to propofol must be expected in patients older than 70 years of age who have various co-morbidities[83].

A study evaluating the safety of sedation with propofol in patients > 90 years of age showed that for upper GI endoscopy, percutaneous endoscopic gastrostomy, colonoscopy, and ERCP, the mean propofol doses used were 22, 24, 46 and 42 mg, respectively. In upper GI endoscopy, the level of sedation and propofol blood concentrations after administration of the drug in the group of patients > 90 years of age corresponded to those resulting from propofol use in middle-aged patients[84].

Finally, Martínez *et al*[85] found that continuous propofol sedation in patients > 80 years of age is generally as safe as in younger patients, although patients > 80 years showed a greater tendency to develop severe oxygen desaturation during the colonoscopy and endoscopic ultrasonography procedures. In general, there were no significant differences in sedation-related complications between the two groups.

Sedation in time-consuming endoscopic procedures

Time-consuming endoscopic procedures, such as ERCP, endoscopic ultrasonography and endoscopic mucosal resection, require sedation for a significantly longer period of time compared with routine upper and lower GI tract endoscopy. Moreover, endoscopic submucosal dissection for early gastric cancer generally lasts much longer than conventional endoscopy and usually requires moderate-to-deep sedation with close surveillance to ensure patient safety, thus increasing the risks related to sedation and analgesia. Therefore, the administration of safe sedation of a satisfactory degree for a longer period of time is necessary.

During recent years, a significant number of papers have been published examining the most suitable and effective drug or combination of drugs for these complex procedures. The data from these clinical studies are discussed below.

Concerning ERCP, a study comparing satisfaction, recovery scores, and safety profiles for ERCP sedation between continuous infusion of propofol and conventional sedation revealed that the continuous infusion of propofol for ERCP under the direction of a gastroenterologist yields no differences in the procedure completion rate and adverse profiles compared with intermittent meperidine and midazolam injection. However, the infusion of propofol does provide a better recovery profile[86].

It is well known that the dose requirements and complications of propofol are less when used in the diluted form than when used in the undiluted form. In a study investigating diluted and undiluted propofol requirements and recovery time in patients undergoing ERCP, it was shown that the requirements in both groups were comparable, although the incidence of sedation-related hypotension was lower in the diluted group[87].

An interesting study showed that patient-controlled sedation with propofol/remifentanil seems to be a well-accepted sedation regimen for ERCP. Additionally, the study showed that anaesthesiologist-managed propofol sedation using constant propofol infusion is associated with deep sedation without any impact on the degree of patient or gastroenterologist satisfaction[88].

EUS and ERCP can be safely performed under conscious sedation on the same day with minimal adverse events. However, combined procedures are associated with higher doses of sedatives and a slightly longer recovery time[89].

Finally, another interesting study suggested that synergistic sedation with an oral dose (7.5 mg) of midazolam 30 min before *i.v.* propofol is given combined with *i.v.* propofol could result in a significant reduction in the dosage of propofol required and in patient anxiety levels before ERCP[27].

With regard to endoscopic ultrasonography, a prospective, randomised study demonstrated that patient-controlled sedation/analgesia with propofol and fentanyl is a more effective and safe technique compared with midazolam and pethidine, resulting in a high level of satisfaction of both patients and endoscopists[21].

Concerning endoscopic mucosal dissection (ESD) for early gastric cancer, a recent study in Japan revealed that ESD performed under sedation using continuous propofol infusion for early gastric cancer was as safe as ESD performed using intermittent midazolam injection. Moreover, patients treated with continuous propofol administration experienced a quicker recovery time than those treated with midazolam[90].

A more recent study, also from Japan, confirmed the results of the study by Kiriyama *et al*[90] and suggested that propofol is as safe and effective as midazolam during ESD. Despite these promising results, sedation guidelines for the use of propofol in early gastric cancer are needed[91].

Among the newer drugs used for sedation and analgesia during GI endoscopy, dexmedetomidine has been used in patients with early gastric cancer undergoing ESD. In a randomised study of 90 patients who underwent ESD treatment, sedation was achieved with either dexmedetomidine (3.0 μg/kg *i.v.* per hour over 5 min, followed by continuous infusion at 0.4 μg/kg per hour), propofol, or midazolam. It was shown that none of the dexmedetomidine-sedated patients developed a significant reduction in the oxygen saturation level. The rate of effective sedation was significantly higher in the dexmedetomidine group compared with the other two groups. It seems, therefore, that sedation with dexmedetomidine is safe and effective in patients with gastric tumours who are undergoing ESD[49].

ADVERSE EVENTS DURING SEDATION FOR ENDOSCOPY

Sedation is usually safe; however, complications may occur, although in various proportions depending on a

number of factors, including the type, dose and mode of administration of sedative drugs, as well as the patient's age and underlying chronic disorders. A large number of side effects, including hypotension, desaturation, bradycardia, hypertension, arrhythmia, aspiration, respiratory depression, vomiting, cardiac arrest, respiratory arrest, angina, hypoglycaemia, and/or allergic reaction, have been reported.

A study in patients submitted to colonoscopic examination showed that midazolam combined with propofol appeared to influence the pulse rate and blood pressure at a significantly higher rate than a combination with fentanyl or midazolam alone. The combination with fentanyl had a significantly lower effect on pulse rate and blood pressure[32].

Prolonged hypoxemia (oxygen saturation of < 90% for ⩾ 15 s) and apnoea (lack of respiratory activity for ⩾ 15 s) are not uncommon during moderate sedation for endoscopy. In a related study, it was noted that hypoxemia usually occurs within 5 min of medication administration or endoscope intubation and that only 1/3 of all apnoea/abnormal ventilation events lead to hypoxemia. Additionally, the total dose of meperidine/fentanyl and the total dose of midazolam are predictors of apnoea[92].

Dreaming is commonly reported after propofol-based sedation. In a relevant study, the per cent of patients reporting dreaming was 19%. It seems that this phenomenon appeared more frequently in patients who received high doses of propofol and in patients who had lower bispectral index values during sedation[93].

In a study of 17 999 endoscopic procedures performed over 8 years, the authors concluded that deep sedation during endoscopic procedures is safe[94]. They noted that adverse events occurred in a small proportion of patients (4.5%) and that six complications, i.e., hypotension, desaturation, bradycardia, hypertension, arrhythmia, and aspiration, occurred in more than 0.1% of patients.

Conigliaro *et al*[95] found a percentage of 0.47% of complications related to endoscopy in patients undergoing colonoscopy when sedation was used as recommended by the guidelines.

The administration of propofol as a sedative agent in GI endoscopy resulted in a significant reduction in mean arterial pressure compared with pre-intervention values, although severe hypotension (systolic blood pressure < 60 mmHg) was noted in 0.5% of patients. Oxygen saturation decreased from 96.5% to 94.4%, although a critical decrease in oxygen saturation (< 90%) was documented in only 2.4% of patients[28].

In cirrhotic outpatients undergoing upper GI endoscopy, sedation with a combination of propofol plus fentanyl or midazolam plus fentanyl revealed no significant differences in the rate of complications between the two groups (14% *vs* 7.3%). In this study, both sedation schemes appeared to be safe[69].

Another study revealed no significant differences in complication rates between propofol deep sedation and meperidine/midazolam administered for moderate sedation. In this study, the complication rate with propofol was 0.60%, compared with 1% in the historical case-control (meperidine/midazolam moderate sedation) group[96]. Among the 324 737 unique procedures performed in patients under conscious sedation, unplanned events were reported in 1.4% of the procedures, 0.9% of which were associated with unplanned cardiopulmonary events[97]. Ljubicić *et al*[98] observed a decrease in oxygen saturation to < 85% and a temporary decrease in heart rate to < 50 beats/min in 5.5 and 11.8% of patients receiving propofol for endoscopy, respectively. Finally, Amornyotin *et al*[87] noted significantly different overall complication rates of 18.2% and 42.9%, respectively, between patients receiving diluted or undiluted propofol for ERCP. Significant differences were also noted in the overall rate of cardiovascular events.

In conclusion, sedation in GI endoscopic procedures, even in time-consuming procedures, seems to be safe. The rate of complications, either cardiovascular or respiratory, could be characterised as "acceptable", provided that all prevention measures have been adopted and the endoscopy staff is suitably equipped, properly trained in the handling of possible complications, and ready to immediately apply rescue measures. Table 4 lists the main sedation-related adverse events occurring during endoscopy in clinical trials, while Table 5 shows the main adverse effects related to the administration of drugs used for sedation in GI endoscopy.

LEGAL ISSUES RELATED TO SEDATION IN GI ENDOSCOPY

Important medical and legal issues regarding sedation have been raised during recent years. Such issues include informed consent of the patient, difficulties in assessing withdrawal of consent in a sedated patient, and the need for sedation monitoring that meets accepted standard of care guidelines[99]. Other controversies possibly related to medico-legal aspects include both the use of propofol and the administration of sedation by anaesthesia personnel. The former controversy is extremely important from a legal point of view if the continuously increasing use of propofol in GI endoscopy by non-anaesth esiologists is taken into account. In a related article, Axon emphasises the possible clinical negligence that could be associated with sedation administration. Interestingly, while the law recognises the desirability of sedation in endoscopy procedures, the facts of a particular case will be scrutinised to determine possible responsibilities of the endoscopist if an adverse outcome occurs[100]. Some questions related to the administration of sedation during GI endoscopy are discussed below.

Should sedation be administered by an endoscopist gastroenterologist or an endoscopist nurse?

The optimal drug for sedation administered by non-anaesthesiologists should have certain properties, such as a

Table 4 Main adverse events related to sedation occurring during endoscopy in clinical trials

Ref.	Drug regimen	Percentage of side effects	Severe hypotension (< 60 mmHg)	Severe desaturation (< 90%)
Ljubicić *et al*[98]	Propofol	17.3% (including bradycardia: 11.8%)		5.5%
Conigliaro *et al*[95]	Midazolam	0.47%		
Gasparović *et al*[28]	Propofol	2.9%	0.5%	2.4%
Sharma *et al*[97]	Cardiopulmonary events	EGD: 0.6%; Colonoscopy: 1.1%; ERCP: 2.1%; EUS: 0.9%		
Nayar *et al*[96]	Propofol deep sedation *vs* moderate sedation	0.6% *vs* 1.0%	0.1%	0.1% (apnoea: 0.3%)
Correia *et al*[69]	Midazolam plus propofol *vs* midazolam plus fentanyl	14% *vs* 7.3%		
Amornyotin *et al*[87]	Diluted *vs* undiluted propofol for deep sedation	18.2% *vs* 42.9%	11.4% *vs* 31.0%	0 *vs* 2.4%
Wang *et al*[32]	Midazolam *vs* midazolam combined with either fentanyl or propofol		Midazolam combined with propofol resulted in hypotension and bradycardia more significantly than a combination with fentanyl or midazolam alone	

EUS: Endoscopic Ultrasound; EGD: Esophagogastroduodenoscopy; ERCP: Endoscopic retrograde cholangiopancreatography.

Table 5 Side effects related to the administration of drugs used for sedation in gastrointestinal endoscopy

Side effect	Midazolam	Propofol	Fentanyl
Hypotension	Yes	Yes	
Hypertension		Yes	
Heart rate alterations	Arrhythmia	Decrease	Arrhythmia
Respiratorydepression	Yes	Yes	Yes (particularly in the elderly)
Apnoea	Yes (in combination with fentanyl)	Yes	Yes (in combination with Midazolam)
Dystonia		Yes	Yes
Priapism		Yes	Yes (very rarely)
Pain on injection		Yes	
Lactic acidosis		Yes	
Intraocular pressure changes		Decrease	
Myoclonic movements		Yes	
Nervous system side effects	Yes (especially in the elderly)	Rare	Yes
Unusual dreams		Yes	
Hypersensitivity	Yes	Yes	Yes (rarely)
Liver damage		Yes	
Amnesia	Yes		
Impairment of cognitive functions - inability to drive safely	Yes		
Paradoxical behaviour	Yes	Yes	
Gastrointestinal effects (nausea, vomiting, hiccups, diarrhoea)	Yes	Yes	Yes
Sexual disinhibition	Yes		
Potential for abuse			Yes
Haemolysis			Yes (slow injection rates and/or mixture in isotonic fluid)

predictable pharmacokinetic profile, rapid onset of action, analgesic and anxiolytic effects, short recovery time, and minimal associated risks, thus making the presence of an anaesthesiologist unnecessary.

There is evidence suggesting that non-anaesthetists can administer sedative drugs, including propofol, safely and effectively in most cases[101].

In the last decade, a number of studies addressed the safety and efficacy of the administration of propofol during GI endoscopy by either physicians or trained nurses[102,103].

With regard to the occurrence of major side effects in these studies, there were no cases of death among patients submitted to endotracheal intubation. In a prospective trial involving 36 743 cases of nurse-administered propofol sedation, the authors concluded that adequately trained nurses and endoscopists can safely administer propofol[104].

Rex *et al*[105], in a safety review of 646 080 (223 656 published and 422 424 unpublished) endoscopist-directed propofol sedation cases, noted that endotracheal intubation and death occurred in 11 and 4 cases, respectively. They concluded that the endoscopist-directed administration of propofol appears to result in a lower mortality

rate than that of traditional sedation with benzodiazepines and opioids and a comparable rate to that of general anaesthesia administered by anaesthesiologists.

Nurse-administered propofol sedation for endoscopic procedures is safe when performed by personnel properly trained in airway handling and sedation with propofol and has considerable advantages compared with conventional sedation for endoscopy[106].

Finally, in a very recent study assessing the current use of propofol during colonoscopy screening in 29 countries, it was found that non-anaesthesiologist-administered propofol was used by 29.9% of respondents in 9 countries. Approximately 2/3 of the other endoscopists reported that they would consider implementing non-anaesthesiologist-administered propofol in low-risk patients. It was also found that propofol, benzodiazepine plus opioids and benzodiazepine alone were used in 45%, 31% and 14% of cases, respectively. Importantly, the main reasons for not considering non-anaesthesiologist-administered propofol implementation were medico-legal issues and cost[107]. We suppose that these issues have not been solved and will continue to be discussed in the future.

Should sedation be administered only by a specialist anaesthesiologist?

It is well established that most of the complications occurring during GI endoscopy, such as hypoxemia, hypoventilation, airway obstruction, apnoea, arrhythmias, hypotension and vasovagal episodes, are not related to the procedure itself, but rather to sedation. A recent trial compared endoscopist-administered propofol sedation for colonoscopy with anaesthetist-administered deep sedation. It was found that endoscopist-administered propofol sedation for colonoscopy yielded a better level of satisfaction and patient willingness to undergo further colonoscopies under the same conditions, as well as fewer side effects than anaesthetist-administered deep sedation[108].

Guidelines concerning the use of propofol have been delivered by most major endoscopic associations worldwide. The guidelines of the Endoscopic Section of the German Society for Digestive and Metabolic Diseases suggest that "for simple endoscopic examinations and in low-risk patients, sedation with propofol should be induced by a properly qualified physician and can then be monitored by an experienced person with appropriate training. The person must not have any other tasks while monitoring the sedation". Furthermore, they suggest that an anaesthesiologist should be required only in patients with a high-risk profile.

Four major United States GI Societies, the American Association for the Study of Liver Disease, American College of Gastroenterology, American Gastroenterological Association and American Society for Gastrointestinal Endoscopy, suggest that the administration of propofol is comparable to that of standard sedation with benzodiazepines by non-anaesthesiologists with respect to their safety and efficacy profile.

The guidelines of the European Society of Gastrointestinal Endoscopy, the European Society of Gastroenterology and Endoscopy Nurses and Associates, and the European Society of Anaesthesiology (produced by 32 individuals from 12 countries, published in 2010) can be summarised as follows:[109] "The consensus suggested that endoscopists and nurses with appropriate training can safely and effectively administer propofol to low-risk patients undergoing endoscopic procedures".

Therefore, the safety profile of non-anaesthesiologist-administered propofol sedation for GI endoscopy seems to be equivalent to that of standard sedation with respect to the risks of hypoxemia, hypotension and bradycardia in ERCP and EUS. Concerning upper and lower GI endoscopy, ERCP and EUS, the time for sedation induction and the recovery time using non-anaesthesiologist-administered propofol sedation are shorter compared with those associated with standard sedation, while non-anaesthesiologist-administered propofol sedation is most likely more cost-effective than standard sedation for ERCP and EUS.

However, the opinion of almost all anaesthesiology societies concerning the use of propofol by non-anaesthesiologists is definitely negative. They emphasise the fact that the manufacturers of propofol restrict its use solely to personnel trained in general anaesthesia and that the United States Food and Drug Administration denied a petition by gastroenterologists seeking the removal of this particular restriction. In a recent consensus statement, the European Society of Anaesthesiology, together with 20 European national anaesthesiology societies in Europe, published new guidelines, entitled "Non-anaesthesiologist Administration of Propofol for Gastrointestinal Endoscopy". They stated that due to its significant risks, propofol should be administered only by those trained in the administration of general anaesthesia[110]. Again, this is a topic of continued debate. International consensus by the major endoscopy societies of the world is urgently needed.

CONCLUSION

Currently, both diagnostic and therapeutic endoscopy is well tolerated and accepted by both patients and endoscopists due to the application of sedation by most centres in the world. During the last 15 years, dramatic changes have occurred in endoscopic procedures, mainly with regard to the sedation techniques and the sophisticated endoscopic instruments and equipment utilised. Today, a large number of drugs are available for achieving successful moderate and deep sedation, and other substances are in the clinical evaluation stage. Moderate sedation using midazolame and an opioid represents the standard method of sedation, although propofol is being increasingly used in many countries. We suggest that the use of this drug will be accepted by an increasing number of endoscopists and that it could become the

preferred sedation agent in the near future. Today, midazolam remains the benzodiazepine of choice, while the most popular opioids are pethidine and fentanyl. Safe sedation in special clinical circumstances, such as in obese, pregnant, and elderly individuals, as well as in patients with chronic lung, renal or liver disease, requires modification of the drug doses used for sedation. It is also crucial for endoscopists to be very familiar with the drug or combination of drugs that they are using in everyday clinical practice. However, the controversy regarding the administration of sedation by an endoscopist, anaesthesiologist or an experienced nurse continues. We emphasise that sedation under the supervision of a properly trained endoscopist could become the standard practice. Due to the legal issues related to the occurrence of unwanted effects of sedation, an updated international consensus regarding the use of sedative agents, especially propofol, is urgently needed.

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ORIGINAL ARTICLE

Age-dependent slowing of enteric axonal transport in insulin-resistant mice

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Abstract

AIM: To investigate retrograde tracer transport by gastric enteric neurons in insulin resistant mice with low or high glycosylated hemoglobin (Hb).

METHODS: Under anesthesia, the retrograde tracer fluorogold was superficially injected into the fundus or antrum using a microsyringe in KK Cg-Ay/J mice prior to onset of type 2 diabetes mellitus (T2DM; 4 wk of age), at onset of T2DM (8 wk of age), and after 8, 16, or 24 wk of untreated T2DM and in age-matched KK/HIJ mice. Six days later, mice were sacrificed by CO_2 narcosis followed by pneumothorax. Stomachs were removed and fixed. Sections from fundus, corpus and antrum were excised and mounted on a glass slide. Tracer-labeled neurons were viewed using a microscope and manually counted. Data were expressed as the number of neurons in short and long descending and ascending pathways and in local fundus and antrum pathways, and the number of neurons in all regions labeled after injection of tracer into either the fundus or the antrum.

RESULTS: By 8 wk of age, body weights of KKAy mice (n = 12, 34 ± 1 g) were heavier than KK mice (n = 17, 29 ± 1 g; F (4, 120) = 4.414, P = 0.002] and glycosylated Hb was higher [KK: (n = 7), 4.97% ± 0.04%; KKAy: (n = 6), 6.57% ± 0.47%; F (1, 26) = 24.748, P < 0.001]. The number of tracer labeled enteric neurons was similar in KK and KKAy mice of all ages in the short descending pathway [F (1, 57) = 2.374, P = 0.129], long descending pathway [F (1, 57) = 0.922, P = 0.341], local fundus pathway [F (1, 53) = 2.464, P = 0.122], local antrum pathway [F (1, 57) = 0.728, P = 0.397], and short ascending pathway [F (1, 53) = 2.940, P = 0.092]. In the long ascending pathway, fewer tracer-labeled neurons were present in KKAy as compared to KK mice [KK: (n = 34), 302 ± 17; KKAy: (n = 29), 230 ± 15; F (1, 53) = 8.136, P = 0.006]. The number of tracer-labeled neurons was decreased in all mice by 16 wk as compared to 8 wk of age in the short descending pathway [8 wk: (n = 15), 305 ± 26; 16 wk: (n = 13), 210 ± 30; F (4, 57) = 9.336, P < 0.001], local antrum pathway [8 wk: (n = 15), 349 ± 20; 16 wk: (n = 13), 220 ± 33; F (4, 57) = 8.920, P < 0.001], short ascending pathway [8 wk: (n = 14), 392 ± 15; 16 wk: (n = 14), 257 ± 33; F (4, 53) = 17.188, P < 0.001], and long ascending pathway [8 wk: (n = 14), 379 ± 39; 16 wk: (n = 14), 235 ± 26; F (4, 53) = 24.936, P < 0.001]. The number of tracer-labeled neurons decreased at 24 wk of age in the local fundus pathway [8 wk: (n = 14), 33 ± 11; 24 wk: (n = 12), 3 ± 2; F (4, 53) = 5.195, P = 0.001] and 32 wk of age in the long descending pathway [8 wk: (n = 15), 16 ± 3; 32 wk: (n = 12), 3 ± 2; F (4, 57) = 2.944, P = 0.028]. The number of tracer-labeled enteric neurons was correlated to final body weight for local fundus and ascending pathways [KK: (n = 34), r = -0.746, P < 0.001; KKAy: (n = 29), r = -0.842, P < 0.001] as well as local antrum and descending pathways [KK (n = 36), r = -0.660, P < 0.001; KKAy (n = 31), r = -0.622, P < 0.001]. In contrast, glycosylated Hb was not significantly correlated to number of tracer-labeled neurons [KK (n = 17), r = -0.164, P = 0.528; KKAy (n

= 16), r = -0.078, P = 0.774].

CONCLUSION: Since uncontrolled T2DM did not uniformly impair tracer transport in gastric neurons, long ascending neurons may be more susceptible to persistent hyperglycemia and low effective insulin.

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Key words: Autonomic neuropathy; Retrograde transport; KKAy mice; Fluorogold; Type 2 diabetes mellitus

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INTRODUCTION

Diabetes mellitus (DM), characterized by persistent hyperglycemia and a deficiency of effective plasma insulin, can result in neuropathy of autonomic neurons. In rodent models of type 1 DM (T1DM) and type 2 DM (T2DM), neuropathies of the parasympathetic vagus nerve[1-3] and enteric neurons located within the wall of the gut[4,5] have been described. Many factors likely contribute to the development of autonomic neuropathy[6,7] including accumulation of sorbitol in nerves[8,9], oxidative stress[10,11], and somal deficiency of neurotrophins[7,12-14].

Neurotrophins, proteins that support the proper functioning of neurons, are retrogradely transported from the innervated organ to the soma by an axonal active transport mechanism. Active axonal transport of proteins in the vagus nerve was impaired in animal models of DM. Early studies using nerve ligation demonstrated reduced axonal transport of opiate receptors[15] and choline acetyltransferase[8] in the vagus nerve of streptozotocin (STZ) rats with partial reversal by immediate treatment with insulin[15] or an aldose reductase inhibitor[8]. Retrograde transport by the vagus nerve of endogenous neurotrophins[8] or experimental neuronal tracers injected into the stomach[16,17] was impaired after 16 or 24 wk of uncontrolled hypoinsulinemia and hyperglycemia in STZ rats. In a rodent model of T2DM, the db/db mouse, axonal transport of acetylcholinesterase[18,19] and choline acetyltransferase[20] in peripheral nerves was impaired, but only after at least 25 wk[18,19], approximately double the time observed in STZ models of T1DM[16,17,21]. To date, active axonal transport of proteins by enteric neurons located within the wall of the gut has not been evaluated in rodent models of DM. We hypothesized that retrograde transport of the neural tracer fluorogold (FG) would be impaired in enteric neurons, but only after prolonged insulin resistance and hyperglycemia.

Coordinated contraction and relaxation of fundus, corpus and antrum is required for normal storage and emptying of gastric contents[22,23]. Enteric neural control of contraction and relaxation of gastric smooth muscle varied by region in rodent models of DM[24,25]. Therefore, tracer transport in ascending and descending enteric neurons innervating the fundus and antrum, respectively, were evaluated.

These studies used an obese mouse model of T2DM, the KKAy mouse. As the result of an agouti mutation on a diabetes susceptible background (KK strain), the KKAy mouse overeats normal chow, accumulates adipose tissue, and develops overt hyperglycemia. Since both mouse groups are insulin resistant[26-28], but only the KKAy mouse group develops persistent hyperglycemia[26], the contribution of overt hyperglycemia to enteric retrograde axonal transport could be determined. We hypothesized that the combination of insulin resistance and overt hyperglycemia in KKAy mice would accelerate the onset of impaired FG transport by enteric neurons as compared to KK mice.

MATERIALS AND METHODS

Animals

Female KK Cg-Ay/J mice (KKAy, strain 2468, n = 60), an obese model of T2DM, and age-matched female KK/HIJ mice (KK, stock number 2106, n = 70), were obtained at 3-6 wk of age (Jackson Laboratories, Bar Harbor, ME, United States). Initially, KKAy mice were screened for onset of T2DM by urine glucose. Since most KKAy mice became diabetic at 8 wk of age, onset of T2DM was defined as 8 wk of age and T2DM was untreated for 8, 16, or 24 wk. Body weight was recorded weekly. At sacrifice, drops of whole blood were used to determine glycosylated hemoglobin (Hb) (A1C Now+® Multi Test A1C System; Bayer, Tarrytown, NY, United States) and stress-induced glucose (BD Logic blood glucose monitor; Becton, Dickinson, and Co., Franklin Lakes, NJ, United States). Urine was drained from the bladder using a syringe and tested for glucose using uristix (Bayer). Urine was considered positive for glucose if glucose was $\geqslant$ 500 mg/dL.

Injection of FG into stomach

Surgeries were performed prior to onset of T2DM (4 wk of age), at onset of T2DM (8 wk of age), and after 8, 16, or 24 wk of untreated T2DM (i.e., 16, 24, and 32 wk of age, respectively). The number of mice at each age is shown in Table 1. Both KKAy and age-matched KK mice surgeries were performed on the same day using the same tracer solution. Surgeries were performed under ketamine (100-150 mg/kg, *ip*; Fort Dodge Animal Health, Fort Dodge, IA, United States) and xylaxine (8 mg/kg, *ip*; Vedco, St. Joseph, MO, United States) anesthesia. The neural tracer FG was used to assess active axonal retrograde transport in enteric neural pathways.

The tracer solution containing 4% FG (hydroxystilbamidine methanesulfonate; Invitrogen Molecular Probes) in dimethyl sulfoxide was superficially injected into the

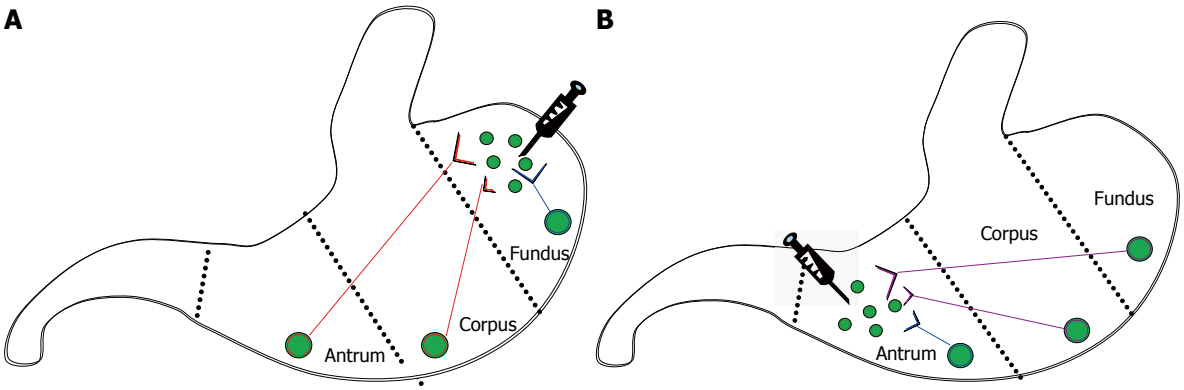

Figure 1 Cartoon of ascending, descending and local enteric pathways in the stomach. Enteric nerve terminals endocytosed the tracer fluorogold (small green circles) and it was transported back to cell bodies (large green circles) located in the fundus, corpus, or antrum. A: The retrograde tracer fluorogold was injected into the fundus. Red neurons were in the ascending pathway while blue neurons were in the local fundic pathway. The approximate distances from fundus injection sites were 0.6, 2.2, and 3.3 cm for fundus, corpus, and antrum sections, respectively; B: The retrograde tracer fluorogold was injected into the antrum. Purple neurons were in the descending pathway while blue neurons were in the local antral pathway. The approximate distances from antrum injection sites were 3.2, 1.1, and 0.4 cm for fundus, corpus, and antrum sections, respectively.

ventral fundus or ventral antrum at 5 sites, 2 μL each, using a microsyringe (702RN, 25 μL; Hamilton, Reno, NV, United States) with replaceable needle (3/8, point style 2, 33 G, Hamilton). An analgesic (buprenorphine, 0.05 mg/kg, *sc*; Hospira, Inc., Lake Forest, IL, United States) was administered immediately after surgery. Ketoprofen (5 mg/kg, *ip*; Fort Dodge Animal Health) was administered 6 h after surgery and the following day.

To promote selective labeling, care was taken to inject the tracer into the gastric wall and avoid leakage of the tracer into the abdominal cavity. Other experiments performed in the lab using the same injection technique have shown that ventral injection of tracer into the stomach wall resulted in unilateral FG labeling of efferent vagal neurons in the brainstem dorsal motor nucleus[29]. If tracer were not confined to the stomach wall, brainstem labeling would be bilateral.

Stomach removal

Six days after tracer injection, mice were sacrificed by CO_2 narcosis followed by pneumothorax. Stomachs were removed, placed in oxygenated Krebs solution, trimmed, opened along the lesser curvature, and emptied of contents. The fundus was cut away from the corpus/antrum. Tissues were stretched and pinned, mucosa side up, in a sylgard-lined Petri dish then fixed overnight at 37 ℃ in 4% paraformaldehyde in 0.1 mol/L PBS. The mucosa was removed. Using a razor blade, the center of each fundus, corpus, and antrum region along the greater curvature was cut out and mounted on a glass slide then coverslipped. The approximate distances of the excised sections from fundus injection sites were 0.6, 2.2, and 3.3 cm for fundus, corpus, and antrum sections, respectively. The approximate distances from antrum injection sites were 3.2, 1.1, and 0.4 cm for fundus, corpus sections, and antrum sections, respectively.

Counting FG-labeled neurons

Tracer-labeled neurons were viewed at 20× using a microscope (Leica DMIL with UV light source; Leica Microsystems Inc., Buffalo Grove, IL, United States) with attached digital camera (MagnaFire; Optronics, Goleta, CA, United States) using the Chroma filter for FG (11006V3 Gold: Ex. 300-390, Em. ≥ 515). Digital images (1280 by 1024 pixels) were 0.327 μm/pixel. Digital images were in 32 bit RGB color at an image resolution of 100 dpi and a computer screen resolution of 92 dpi. Labeled neurons were manually counted with the assistance of ImagePro Plus (version 4.5.0.29, Media Cybernetics, Silver Spring, MD, United States). The numbers of labeled neurons in 20 adjacent images (418.6 μm by 334.8 μm) of the fundus and in 12 adjacent images of the corpus and antrum (total area: 2.8, 1.7, and 1.7 mm^2, respectively) were counted and data were expressed as (1) total number of tracer-labeled neurons in that pathway; and (2) total number of neurons in all three regions labeled after tracer injection into the fundus or antrum.

Description of ascending, descending, and local enteric neural pathways

Six pathways in the stomach were assessed in this study (Figure 1). Enteric neurons in ascending pathways had cell bodies in the corpus (short ascending pathways) or antrum (long ascending pathways) and nerve terminals in the ventral fundus, while those in descending pathways had cell bodies in the fundus (long descending pathways) or corpus (short descending pathways) and nerve terminals in the ventral antrum. Enteric neurons in fundic local pathways had both cell bodies and nerve terminals in the ventral fundus, while those in antral local pathways had both cell bodies and nerve terminals in the ventral antrum.

Statistical analysis

All data were expressed as mean ± SE. Data were statistically analyzed using SPSS (version 18.0, Chicago, IL). Independent factors for analysis of variance included KK or KKAy, age (4, 8, 16, 24, or 32 wk) or pathway (ascending or descending). Both main effects of one

Table 1 Number of mice at each age with tracer injection into the fundus or antrum

Age (wk)	Fundus	Antrum
KK mice		
4	6	6
8	8	9
16	8	7
24	6	8
32	6	6
KKAy mice		
4	6	6
8	6	6
16	6	6
24	6	7
32	5	6

independent factor and interactions of two independent factors were evaluated and Sidak correction was used for post-hoc analysis. The ratios of KK and KKAy mice with positive urine glucose were compared using χ^2. Correlations were performed using Pearson correlation (r), linear regression (r^2) was performed using SigmaPlot (version 12.0, Systat Software, Inc., San Jose, CA). The number inside the bar in the graphs indicates the number of animals in the group. A P value < 0.05 was considered statistically significant with $^aP < 0.05$, $^bP < 0.01$.

RESULTS

Animal characteristics

Body weights of KK and KKAy mice were similar at 4 wk of age (Table 2), then KKAy mice became heavier [interaction: KK/KKAy and age F (4, 120) = 4.414, P = 0.002]. Body weight plateaued in KK and KKAy mice at 24 and 16 wk of age, respectively.

Glucose control was impaired in KKAy mice (Table 2). Glycosylated Hb, an index of average plasma glucose over the previous 40 d for a mouse[30], was elevated in KKAy as compared to KK mice [main effect F (1, 26) = 24.748, P < 0.001] but did not change with age [main effect: F (3, 26) = 1.916, P = 0.152]. For all mice 8 wk of age and older, glycosylated Hb was higher in KKAy mice (6.53 ± 0.31, n = 17) than in KK mice (5.02 ± 0.04, n = 17). The ability to raise blood glucose in response to stress was greater in KKAy as compared to KK mice. Stress-induced hyperglycemia was highest at 16 wk in KKAy mice (535 ± 47, n = 7), but remained steady in KK mice (297 ± 20, n = 9) [interaction: KK/KKAy and age F (4, 67) = 2.609, P = 0.043]. Glucose was detected in the urine of some KKAy mice prior to onset of T2DM, but was not detected in any KK mice.

Tracer-labeled neurons in enteric pathways

Six days after injection of tracer into the fundus or antrum, FG accumulated in the cell bodies of enteric neurons of the fundus, corpus, and antrum. Tracer-labeled enteric neurons of the corpus are shown in Figure 2.

In the long ascending pathway (Figure 3A), significantly fewer FG-labeled enteric neurons were detected in KKAy as compared to KK mice. For both KK and KKAy mice, the number of FG-labeled neurons was reduced by 16 wk of age and reached its lowest value at 24 wk of age.

In the short ascending pathway (Figure 3B), there was a trend for fewer FG-labeled enteric neurons in KKAy as compared to KK mice. By 16 wk of age, the numbers of FG-labeled neurons were reduced as compared to 4 and 8 wk of age. The numbers of FG-labeled neurons remained low at 24 and 32 wk of age.

In the long (Figure 3D) and short (Figure 3E) descending pathways, similar numbers of FG-labeled enteric neurons were detected in KK as compared to KKA^y mice. The total number of FG-labeled enteric neurons reached its lowest value at 24 wk of age for the short descending pathway and 32 wk of age for the long descending pathway.

A similar dominance of ascending projections as compared to descending projections reported in guinea pig stomach[31,32] was observed in this mouse study. At 4 wk of age, more FG-labeled neurons were in the ascending pathways as compared to the descending pathways for KK and KKAy mice [main effect: F (1, 20) = 98.981, P < 0.001; KK: ascending (n = 6), 858 ± 45; descending (n = 6), 397 ± 55; KKAy: ascending (n = 6), 800 ± 37; descending (n = 6), 384 ± 37].

Similar numbers of FG-labeled fundic (Figure 3C) and antral (Figure 3F) enteric neurons were detected in KK as compared to KKAy mice. In the local fundic pathway, the

Table 2 Animal characteristics

	Body weight (g)	HbA1c (%)	Urine glucose[1]
4 wk of age			
KK	19.2 ± 0.4 (n = 12)	< 4.0 (n = 3)	0/6
KKAy	19.9 ± 0.5 (n = 12)	< 4.0 (n = 3)	2/6
8 wk of age			
KK	29 ± 1[d] (n = 17)	5.0 ± 0.0 (n = 7)	0/6
KKAy	34 ± 1[b,d] (n = 12)	6.6 ± 0.5[b] (n = 6)	1/3[1]
16 wk of age			
KK	35 ± 1[d,f] (n = 15)	5.1 ± 0.1 (n = 3)	0/13
KKAy	43 ± 1[b,d,f] (n = 12)	7.5 ± 0.7[b] (n = 3)	8/11[1]
24 wk of age			
KK	40 ± 1[d,f,h] (n = 14)	5.3 (n = 2)	0/12
KKAy	45 ± 1[b,d,f] (n = 13)	7.0 ± 0.9[b] (n = 3)	7/11[1]
32 wk of age			
KK	39 ± 2[d,f] (n = 12)	5.0 ± 0.1 (n = 5)	0/9
KKAy	47 ± 1[b,d,f] (n = 11)	5.6 ± 0.5[b] (n = 5)	3/11

[1]Number of mice with urine glucose ⩾ 500 mg/dL out of the total number of mice tested for urine glucose. [b]P < 0.01 *vs* KK; [d]P < 0.01 *vs* 4 wk; [f]P < 0.01 *vs* 8 wk; [h]P < 0.01 *vs* 16 wk. HbA1c: Haemoglobin A1c.

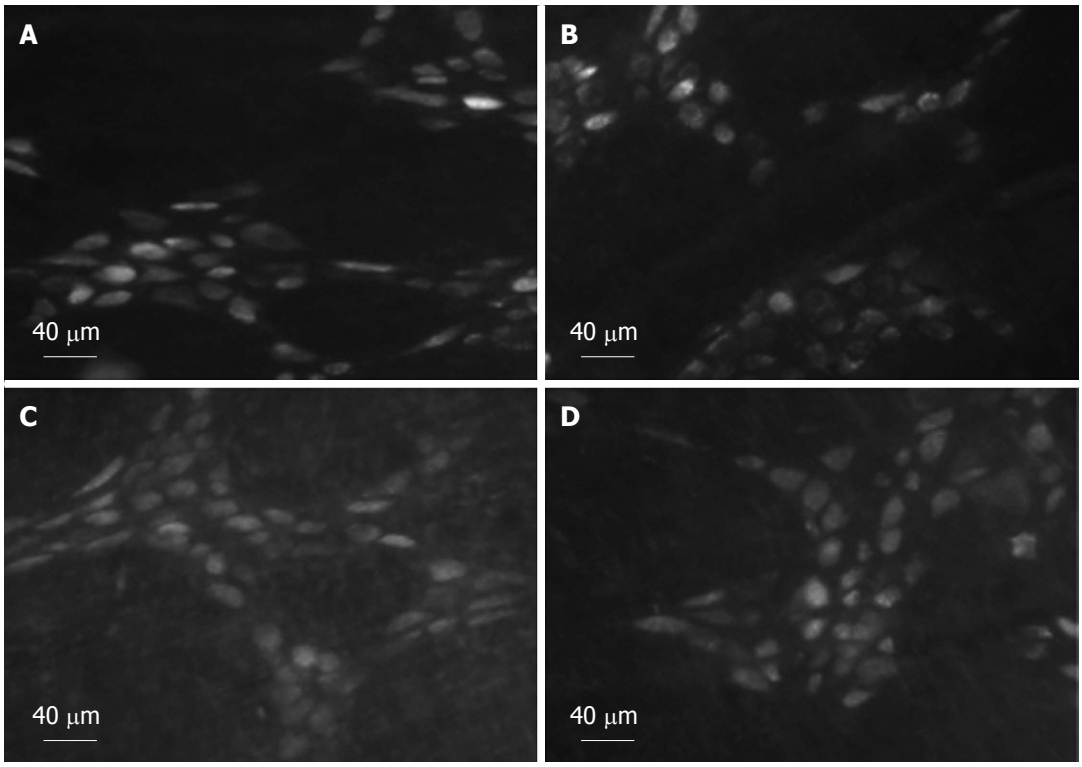

Figure 2 Fluorogold-labeled enteric neurons in gastric corpus. A: Fluorogold-labeled ascending enteric neurons in KK mice; B: Fluorogold-labeled ascending enteric neurons in KKAy mice; C: Fluorogold-labeled descending enteric neurons in KK mice; D: Fluorogold-labeled descending enteric neurons in KKAy mice. The brightness of neurons varied depending on the amount of fluorogold transported to the cell body, but most neurons in the ganglia were labeled with fluorogold.

total number of FG-labeled enteric neurons was variable, reaching its highest value at 8 wk and its lowest value at 24 wk of age. In contrast, in the antral pathway, the total number of FG-labeled enteric neurons was decreased by 16 wk in KK and KKA^{y} mice.

Correlation of FG-labeled neurons with body weight or blood glucose

The total number of FG-labeled neurons in all sections after injection into either the fundus or antrum was significantly correlated to mouse final body weight, but not stress-elevated blood glucose (Figure 4). As KK mice aged and body weight increased, the total number of enteric neurons labeled by fundus injection (Figure 4A) decreased (n = 34, r = -0.746, P < 0.001). In the heavier KKAy mice, the correlation was stronger (n = 29, r = -0.842, P < 0.001). As a result of injection into the antrum (Figure 4B), the total number of FG-labeled neurons similarly decreased with body weight in both groups of mice [KK (n = 36), r = -0.660, P < 0.001; KKAy (n = 31), r = -0.622, P < 0.001]. In contrast, stress-elevated blood glucose was not correlated with the number of FG-labeled neurons [fundus injection (Figure 4C): KK (n = 17), r = -0.097, P = 0.712; KKAy (n = 15), r = 0.328, P = 0.232; antrum injection (Figure 4D): KK (n = 25), r = 0.174, P = 0.407; KKAy (n = 20), r = 0.308, P = 0.187]. In addition, HbA1c was not correlated to number of FG-labeled neurons for antrum injection [KK (n = 17), r = -0.164, P = 0.528; KKAy (n = 16), r = -0.078, P = 0.774]. Data was not available for fundus injection.

DISCUSSION

The principal findings of these studies were that (1) insulin resistance, in the absence of persistent hyperglycemia, slowed retrograde FG transport in KK mice at 16 wk of age, the approximate age of onset of insulin resistance[26-28]; and (2) persistent hyperglycemia, as indicated by elevated HbA1c, significantly slowed tracer transport in long ascending pathways in KKAy as compared to KK mice, but the age of onset was unchanged. Persistent hyperglycemia did not significantly accelerate the age of onset of neuropathy in KKAy as compared to KK mice.

Reduced numbers of FG-labeled neurons may be due to many factors related to enteric neuropathy. Enteric neurons undergoing neuropathy may take up less tracer because of limited endocytosis[33]. Retrograde transport of tracer may be impaired due to glycosylation of axonal cytoskeletal proteins[34-36]. The density of nerve terminals in the tracer injection field may be reduced due to degeneration of nerve terminal processes[3,37,38] or to low rates of neuronal apoptosis[1,39] as a consequence of metabolic disturbances[40]. In addition, impaired active axonal transport of growth factors to the neuronal soma, including insulin and neurotrophins[11,14,41-44], may both promote and exacerbate neuropathy[6,7].

Specific mechanism(s) in distinct populations of neurons may contribute more significantly to neuropathy. Many factors play a role in the development of diabetic neuropathy[6,7,45], but treatments targeting a single mechanism do not prevent neuropathy of all autonomic neu-

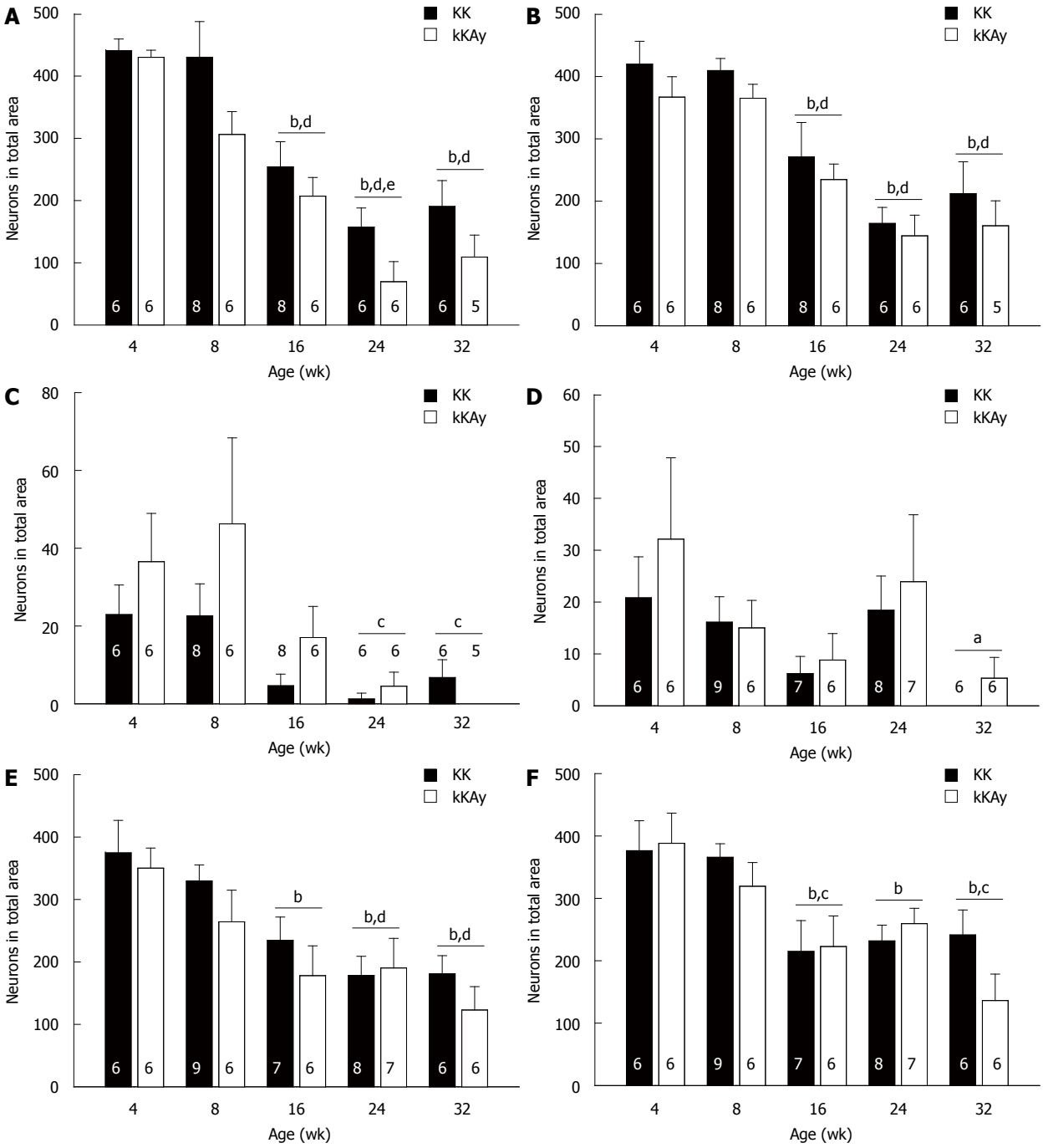

Figure 3 Number of fluorogold-labeled neurons in enteric neural pathways in 4-32 wk old KK and KKAy mice. A: Long ascending pathway: The number of fluorogold (FG)-labeled neurons was significantly decreased in KKAy as compared to KK mice [main effect: F (1, 53) = 8.136, P = 0.006]. The numbers of FG-labeled neurons began to decrease with age in KK and KKAy mice at 16 wk of age with the fewest neurons detected at 24 wk of age [main effect: F (4, 53) = 24.936, P < 0.001]; B: Short ascending pathway: There was a trend for the numbers of FG-labeled neurons to be reduced in KKAy as compared to KK mice [main effect: F (1, 53) = 2.940, P = 0.092]. The number of FG-labeled neurons decreased with age in KK and KKAy mice [main effect: F (4, 53) = 17.188, P < 0.001]. By 16 wk of age, the numbers of FG-labeled neurons were reduced and remained low; C: Fundus: Similar numbers of FG-labeled neurons were observed in KK and KKAy mice [main effect: F (1, 53) = 2.464, P = 0.122]. In KK and KKAy mice, the numbers of labeled FG neurons were highest at 8 wk and lowest at 24 and 32 wk [main effect: F (4, 53) = 5.195, P = 0.001]; D: Long descending pathway: Similar numbers of FG-labeled neurons were observed in KK and KKAy mice [main effect: F (1, 57) = 0.922, P = 0.341]. By 32 wk of age, the numbers of FG-labeled neurons were reduced as compared to younger ages [main effect: F (4, 57) = 2.944, P = 0.028]; E: Short descending pathway: Similar numbers of FG-labeled neurons were observed in KK and KKAy mice [main effect: F (1, 57) = 2.374, P = 0.129]. The numbers of FG-labeled neurons began to decrease in KK and KKAy mice at 16 wk of age with the fewest neurons detected at 24 wk of age [main effect: F (4, 57) = 9.336, P < 0.001]; F: Antrum: Similar numbers of FG-labeled neurons were observed in KK and KKAy mice [main effect: F (1, 57) = 0.728, P = 0.397]. By 16 wk of age, the numbers of FG-labeled neurons were reduced as compared to 4 and 8 wk of age [main effect: F (4, 57) = 8.920, P < 0.001]. ^{a}P < 0.05, ^{b}P < 0.01 *vs* 4 wk; ^{c}P < 0.05, ^{d}P < 0.01 *vs* 8 wk; ^{e}P < 0.05 *vs* 16 wk.

rons[46-48]. In this study, long ascending enteric neurons, but not long descending enteric neurons, displayed significantly slower tracer transport in KKAy as compared to KK mice despite similar axonal lengths. Ascending enteric neurons are predominantly cholinergic[31,49-51]. Impaired axonal transport has been reported for acetylcho-

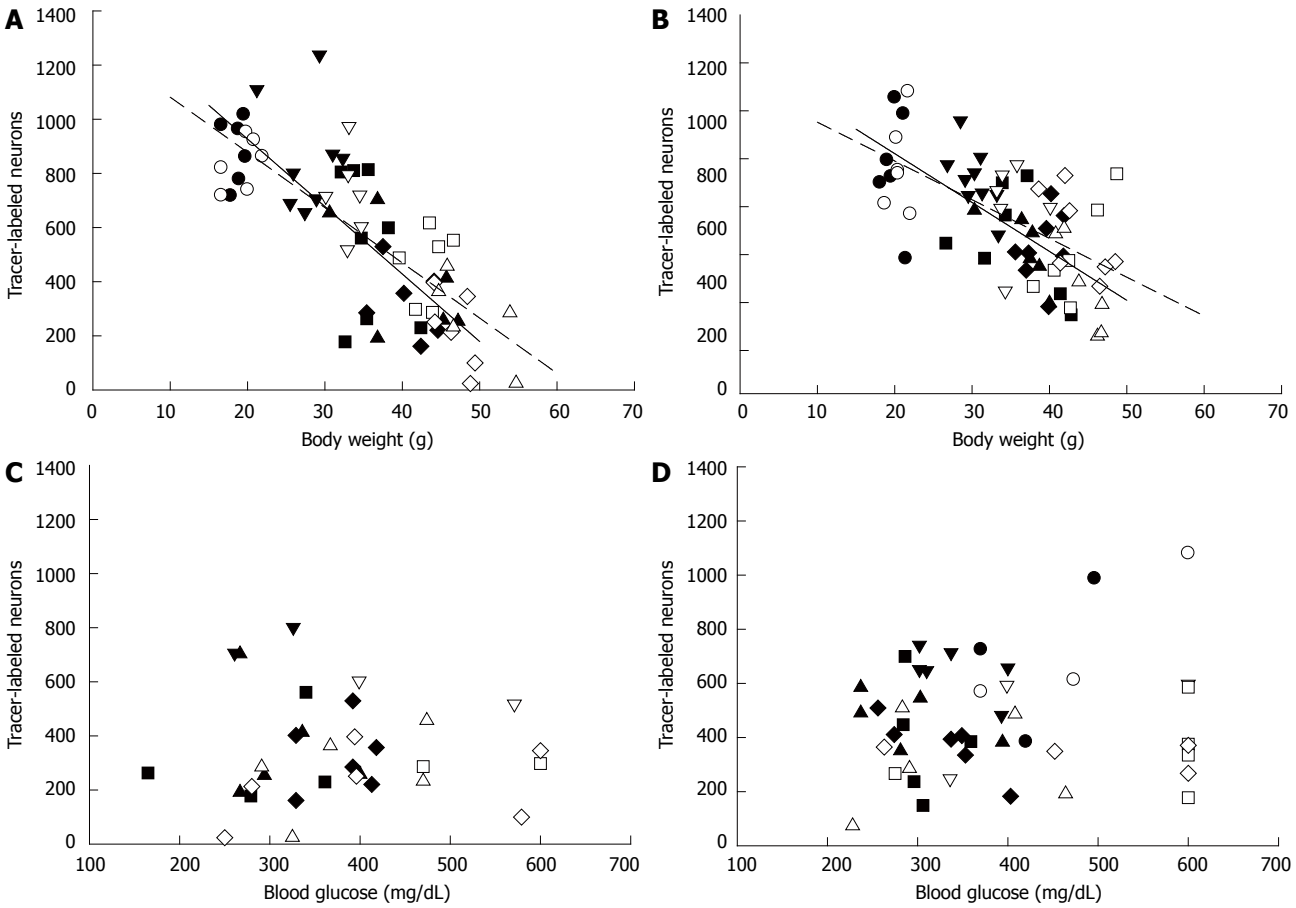

Figure 4 Correlation of body weight or stress-elevated blood glucose with number of tracer-labeled enteric neurons. A: Body weight was correlated with number of neurons labeled by fundus injection (KK mice, r^2 = 0.4362; KKAy mice, r^2 = 0.3868); B: Body weight was correlated with number of neurons labeled by antrum injection (KK mice, r^2 = 0.5565; KKAy mice, r^2 = 0.7087); C: Stress-elevated blood glucose was not correlated with number of neurons labeled by fundus injection (KK mice, r^2 = 0.0093; KKAy mice, r^2 = 0.108); D: Stress-elevated blood glucose was not correlated with number of neurons labeled by antrum injection (KK mice, r^2 = 0.0301; KKAy mice, r^2 = 0.0947). Legend: Black symbols and solid line, KK mice; white symbols and dashed line, KKAy mice; circle, 4 wk; inverted triangle, 8 wk; square, 16 wk; diamond, 24 wk; upright triangle, 32 wk.

linesterase[18,19] and muscarinic receptors[15] and debated for choline acetyltransferase[8,20,43,52] in rodent models of DM. Hence, active tracer transport in this subpopulation of autonomic neurons with a cholinergic phenotype may be more susceptible to combined persistent hyperglycemia and low effective insulin.

Age-related degeneration of axonal branches[37,40] may contribute to the reduction in the number of FG-labeled neurons observed in older as compared to younger mice. In young NMRI mice, the number of myenteric neurons per ganglion section did not change between 1 and 3 mo of age[53]. Similarly, in this study, neuron density was unchanged from 4 to 8 wk of age. In rats, gastric myenteric neurons have been reported to be less susceptible to age-related degeneration than intestinal myenteric neurons[54,55]. The density of gastric myenteric neurons was unchanged until 27 mo of age, when density was reduced in the forestomach, but not corpus or antrum[55]. In this study, the initial reduction in FG labeled neurons was observed at 16 wk of age in KK and KKA^y mice. Hence, in mice with insulin resistance, the reduced delivery of target organ trophic factors back to the cell soma may be associated with increased susceptibility of enteric neurons, especially those with cholinergic phenotype[54], to age-related degeneration[37,56].

A heavier body weight, reflecting a higher degree of adiposity and therefore insulin resistance, contributed more strongly to enteric neuropathy than severity of hyperglycemia. The correlation of total FG-labeled neurons with body weight was stronger for heavier KKAy as compared to lighter KK mice. In contrast, the severity of hyperglycemia was not correlated to total FG-labeled neurons. Other studies have also reported that insulin deficiency, but not hyperglycemia, was associated with severity of peripheral neuropathy[14,38,57-59].

An increase in stomach size may result in a lower density of nerve terminals in the injection field for FG endocytosis and subsequent reduction in FG labeling. The weights of stomachs from 16 wk old KK and KKAy mice were similar [personal observation, KK (n = 4), 277 ± 40 mg; KKAy (n = 4), 263 ± 33 mg, P = 0.80] suggesting that stomachs from KKAy mice were not hypertrophied.

Insulin resistance itself in the absence of persistent hyperglycemia slowed tracer transport in 16-wk-old KK mice. The addition of persistent hyperglycemia in KKAy mice further slowed tracer transport in only long ascend-

ing enteric neurons without accelerating the age of onset. Slowed active tracer transport in enteric neurons implies that retrograde transport of endogenous neurotrophins required for maintenance of normal nerve function may also be impaired[6,21,33,60]. Given the codependence of neurons and the target tissue on neurotrophins to preserve organ function[6], neuropathy resulting from impaired retrograde transport of neurotrophins may result in imprecise coordination of gastric regions by ascending and descending inhibitory and excitatory enteric reflexes[61,62], thereby contributing to asynchronous gastric motor function[63,64].

COMMENTS

Background

Diabetes mellitus (DM), characterized by persistent hyperglycemia and a deficiency of effective plasma insulin, can result in neuropathy of autonomic neurons. In rodent models of type 1 DM (T1DM) and type 2 DM (T2DM), neuropathies of the parasympathetic vagus nerve and enteric neurons located within the wall of the gut have been described. Many factors likely contribute to the development of autonomic neuropathy including accumulation of sorbitol in nerves, oxidative stress, and somal deficiency of neurotrophins.

Research frontiers

Under anesthesia, the retrograde tracer fluorogold was superficially injected into the fundus or antrum using a microsyringe in KK Cg-Ay/J mice prior to onset of T2DM (4 wk of age), at onset of T2DM (8 wk of age), and after 8, 16, or 24 wk of untreated T2DM and in age-matched KK/HlJ mice. Six days later, mice were sacrificed by CO_2 narcosis followed by pneumothorax. Data were expressed as the number of neurons in short and long descending and ascending pathways and in local fundus and antrum pathways, and the number of neurons in all regions labeled after injection of tracer into either the fundus or the antrum.

Innovations and breakthroughs

The principal findings of these studies were that (1) insulin resistance, in the absence of persistent hyperglycemia, slowed retrograde fluorogold transport in KK mice at 16 wk of age, the approximate age of onset of insulin resistance; and (2) persistent hyperglycemia, as indicated by elevated HbA1c, significantly slowed tracer transport in long ascending pathways in KKAy as compared to KK mice, but the age of onset was unchanged. Persistent hyperglycemia did not significantly accelerate the age of onset of neuropathy in KKAy as compared to KK mice.

Applications

Insulin resistance itself in the absence of persistent hyperglycemia slowed tracer transport in 16-wk-old KK mice. The addition of persistent hyperglycemia in KKAy mice further slowed tracer transport in only long ascending enteric neurons without accelerating the age of onset. Slowed active tracer transport in enteric neurons implies that retrograde transport of endogenous neurotrophins required for maintenance of normal nerve function may also be impaired.

Peer review

The manuscript is formally well written and figures are clear and understandable. Experimental design and methodology are suitable for publication.

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ORIGINAL ARTICLE

Combined early fluid resuscitation and hydrogen inhalation attenuates lung and intestine injury

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Abstract

AIM: To study the effects of combined early fluid resuscitation and hydrogen inhalation on septic shock-induced lung and intestine injuries.

METHODS: Wistar male rats were randomly divided into four groups: control group (Group A, *n* = 15); septic shock group (Group B, *n* = 15); early fluid resuscitation-treated septic shock group (Group C, *n* = 15); and early fluid resuscitation and inhalation of 2% hydrogen-treated septic shock group (Group D, *n* = 15). The activity of hydroxyl radicals, myeloperoxidase (MPO), superoxide dismutase (SOD), diamine oxidase (DAO), and the concentration of malonaldehyde (MDA) in the lung and intestinal tissue were assessed according to the corresponding kits. Hematoxylin and eosin staining was carried out to detect the pathology of the lung and intestine. The expression levels of interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α in lung and intestine tissue were detected by enzyme-linked immunosorbent assay method. The expression levels of Fas and Bcl2 in lung tissues were determined by immunohistochemistry and Western blotting.

RESULTS: Septic shock elicited a significant increase in the levels of MDA (10.17 ± 1.12 nmol/mg protein *vs* 2.98 ± 0.64 nmol/mg protein) and MPO (6.79 ± 1.02 U/g wet tissue *vs* 1.69 ± 0.14 U/g wet tissue) in lung tissues. These effects were not significantly decreased by Group C pretreatment, but were significantly reduced by Group D pretreatment (MDA: 4.45 ± 1.13 nmol/mg protein *vs* 9.56 ± 1.37 nmol/mg protein; MPO: 2.58 ± 0.21 U/g wet tissue *vs* 6.02 ± 1.16 U/g wet tissue). The activity of SOD (250.32 ± 8.56 U/mg protein *vs* 365.78 ± 10.26 U/mg protein) in lung tissues was decreased after septic shock, and was not significantly increased by Group C pretreatment, but was significantly enhanced by Group D pretreatment (331.15 ± 9.64 U/mg protein *vs* 262.98 ± 5.47 U/mg protein). Histological evidence of lung hemorrhage, neutrophil infiltration and overexpression of IL-6, IL-8, and TNF-α was observed in lung tissues, all of which were attenuated by Group C and further alleviated by Group D pretreatment. Septic shock also elicited a significant increase in the levels of MDA, MPO and DAO (6.54 ± 0.68 kU/L *vs* 4.32 ± 0.33 kU/L) in intestinal tissues, all of which were further increased by Group C, but significantly reduced by Group D pretreatment. Increased Chiu scoring and overexpression of IL-6, IL-8 and TNF-α were observed in intestinal tissues, all of which were attenuated by Group C and further attenuated by Group D pretreatment.

CONCLUSION: Combined early fluid resuscitation and hydrogen inhalation may protect the lung and intestine of the septic shock rats from the damage induced by oxidative stress and the inflammatory reaction.

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Key words: Early fluid resuscitation; Inhalation of hydrogen gas; Septic shock; Lung; Intestine; Oxidative damage

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INTRODUCTION

Septic shock is a common complication of infection, severe trauma, and major operation in patients. Septic shock is an autoimmune injury of the body, and the pathogenesis is very complicated. The immunocyte is activated in patients with septic shock, and the respiratory burst creates massive amounts of reactive oxygen species (ROS) which cannot be cleaned by the antioxidant defense systems[1,2]. Oxidative stress induced by ROS can increase lipid peroxidation, and can also increase the permeability of the alveolar epithelial cells by destroying the cell membrane. Simultaneously, the aggregation of neutrophilic leukocytes in the lung further induces the respiratory burst. The imbalance of antioxidant defense systems against oxidative stress then further damages the alveolar epithelial cells.

In order to ensure the blood supply of major organs, including the heart and brain, in patients with septic shock, blood flow is redistributed. There is a significant decrease in blood flow of the gastrointestinal tract which induces severe ischemia, hypoxia, and reperfusion injury. Inflammatory factors are activated which induce further injury in the gastrointestinal tract, and may consequently produce multiple organ dysfunction syndrome (MODS)[3]. Despite recent advances in antibiotic therapy and intensive care, sepsis is still considered to be one of the most common causes of death in intensive care units. The main therapy for septic shock is hemostasis and fluid resuscitation which can prevent MODS by improving hemodynamics[4]. However, fluid resuscitation is not effective in microcirculation disturbance and hypoxia. So, new and effective therapies for septic shock should be developed to improve the patient outcomes.

Recently, it has been suggested that molecular hydrogen exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radicals (•OH, the most cytotoxic ROS) and effectively protects against organ damage[5-7]. Xie *et al*[1,8,9] also report that hydrogen inhalation can significantly decrease levels of oxidative product, increase activities of antioxidant enzymes, and reduce levels of high-mobility group box 1 in serum and lung tissue, thus improving the survival rate of mice with sepsis. However, there are no reports combining fluid resuscitation and hydrogen inhalation as treatment for septic shock. Therefore, the present study was designed to investigate the protective effects of combined early fluid resuscitation and 2% hydrogen on the lung and intestine in a lipopolysaccharide (LPS)-induced septic shock rat model.

MATERIALS AND METHODS

Animals and chemicals

Male Wistar rats weighing 180-200 g were used for the study. They were obtained from the Laboratory Animal Center (China Medical University, Shenyang, China). LPS (L-2880 from *Escherichia coli* serotype 055:B5) was obtained from Sigma Chemical Co. (St. Louis, MO, United States). The •OH, myeloperoxidase (MPO), superoxidase dismutase (SOD), and malonaldehyde (MDA) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kits, rabbit polyclonal anti-Bcl-2 and rabbit polyclonal anti-Fas were purchased from Wuhan Boster Bio-engineering Limited Company (Wuhan, China).

Experimental design

Sixty male Wistar rats were divided into four groups randomly, 15 rats in each: control group (Group A); septic shock group (Group B); early fluid resuscitation + septic shock group (Group C); and early fluid resuscitation + inhalation of 2% hydrogen + septic shock group (Group D). Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg) and tracheally ventilated using a Colombus ventilator (HX-300, TaiMeng Technologies, China), with a respiratory frequency of 100 breaths/min and a tidal volume of 10 mL/kg. The inhaled gas used for Group D was a hydrogen-air mixture (2% hydrogen in air), and the inhaled gas for other groups was room air. Left common carotid artery was cannulated to monitor the heart rate and mean arterial pressure (MAP). The femoral vein was cannulated for drug administration and fluid resuscitation. An electric heater was used to maintain body temperature. Half an hour after the rats reached a stable status, except for the rats in Group A, LPS was administered at a dose of 15 mg/kg by slow intravenous injection (not less than 2 min) to establish the septic shock rat model. Then, 0.2 mL saline was used to ensure that the LPS remaining in the syringe was completely injected[10]. Group A rats received intravenous injections of the same volume of saline. Fluid challenge (10 mL/kg per 15 min) was performed when MAP or arterial blood flow decreased to 80% of baseline values. If the percent change in pulse pressure > 13% with a persistent decrease in MAP or arterial blood flow, the fluid challenge was repeated. Norepinephrine was introduced when MAP remained low despite a normal percent change in pulse pressure (< 13%). Norepinephrine concentration was either progressively increased or decreased between 0.5 and 6 μg/kg.min, as needed, to maintain the MAP at ± 10% of baseline value[10]. Vital signs, fluid volume and the amount of norepinephrine were also recorded.

Preparation of lung tissues

The rats were sacrificed 2 h after the septic shock rat model was established. Arterial blood (1 mL) was collected for assessing the arterial blood gases. The heart and lung were removed through a thoracotomy and the dorsal lobe of the right lung was cut into three blocks (0.1 cm × 0.1 cm × 0.1 cm). The excess liquid of the first block was drained by the filter paper, and wet weight (W) was recorded. Then the same block was dried in a drying oven at a temperature of 70 ℃ for 24 h to measure dry weight (D). The second block was fixed with 4% paraformaldehyde at a temperature of 4 ℃, and then, the block was embedded in paraffin and sliced for hematoxylin and eosin (HE) staining and immunohistochemical study. The other lung tissue block was frozen at -70 ℃ for Western blotting analysis. The right hilum was ligated, and 2 mL of saline solution was used for the left lung lavage, and about 3.0-3.6 mL of bronchial alveolar lavage fluid (BALF) was collected. The BALF was centrifuged at 1500 × *g* for 4 min, and the supernatant was collected and stored at -20 ℃ for inflammatory mediator analysis.

Preparation of small intestine tissues

The rats were sacrificed 2 h after the septic shock rat model was established. Arterial blood (1 mL) was collected for assessing the arterial blood gases. Another 4 mL arterial blood was centrifuged at 2000 rpm for 10 min, and the supernatant serum was collected and stored at -20 ℃. A laparotomy was performed, two small intestinal segments were resected from 10 cm distal to the ligament of Treitz, and the intestinal segments were gently washed using saline rinse cooled to 4 ℃. These two small intestine segments (cut to 1 cm × 1 cm) were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde, respectively. The other small intestine segments used for assessing the oxidative stress were frozen at -70 ℃.

Determination of oxidative damage

Small intestinal tissues and lung tissues were homogenated, centrifuged at 12 000 × *g* for 20 min, and the supernatant serum was collected. The OH, MDA, SOD, and MPO activities were measured according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Measurement of serum diamine oxidase activity

The frozen serum was thawed on ice, and centrifuged at 1000 × *g* for 15 min at 4 ℃. The serum diamine oxidase (DAO) activity was detected with dianisidine developer, and the serum DAO activity assay was performed according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

HE staining of tissues

Both the small intestinal tissue and lung tissues were fixed with 4% paraformaldehyde for more than 24 h. After dehydrating and embedding, the tissues were cut into 5 μm slices. The slices were stained with HE. The pathogenesis of lung injury and small intestinal mucosal injury was observed under microscope. The intestinal mucosal injury was evaluated by the Chiu scoring system. The Chiu's score was graded as: Grade 0: normal mucosa; Grade 1: formation of subepithelial detachments at the tip of the villi with capillary congestion; Grade 2: subepithelial detachments exert a moderate amount of upward push on the mucosal epithelium; Grade 3: large subepithelial detachments exert a massive amount of upward push on the mucosal epithelium along the villi and a few denuded villus tips are observed; Grade 4: the villi are denuded to the level of lamina propria and dilated capillaries; Grade 5: presence of ulceration, disintegration of lamina propria, and hemorrhage[11]. Ten fields for each sample were observed, and the average score was recorded as the pathology score of the small intestinal tissue.

Changes in the organelles of small intestine

The small intestine segments were fixed with 2.5% glutaraldehyde. After dehydration, the samples were embedded in epoxy resins, and then were cut into ultrathin sections. The changes in the organelles were observed under transmission electron microscope.

Measurement of serum inflammatory mediators

The frozen serum was thawed on ice, and centrifuged at 1000 × *g* for 15 min at 4 ℃. Levels of IL-6, IL-8 and TNF-α were detected by ELISA according to the manufacturer's instructions (Wuhan Boster Bio-engineering Limited Company, Wuhan, China).

Immunohistochemical study

Tissue sections (slice thickness 3 μm) were dried for 4 h at 60 ℃-65 ℃, and then were deparaffinized and rehydrated. Sections were immersed in phosphate buffered saline (PBS) three times (5 min/time). Then antigen retrieval was performed followed by cooling at room temperature. The slides were immersed in PBS three times (5 min/time). After that, the sections were incubated with methanol containing 3% hydrogen peroxide for 20 min, and then were left at room temperature for 10 min followed by washing with PBS three times (5 min/time). The slides were blocked with normal goat serum solution at room temperature for 10 min, and the superfluous serum surrounding slides was wiped off. After that, the slides were incubated with primary antibody (50 μL) at 4 ℃ overnight. After washing with PBS three times (5 min/time), sections were incubated in horseradish peroxidase-polymer goat anti-Ms/Rb immunoglobulin G at room temperature for 20 min followed by rinsing in PBS three times (5 min/time). The slides were developed with 3,3'-diaminobenzidine solution for about 3-5 min, rinsed with tap water, and then counterstained with hematoxylin. Acid alcohol solution (1%) was used for differentiation for 1 s, followed by rinsing with tap water for 10-15 min. Finally, specimens were dehydrated and mounted, and examined with a microscope equipped with an image analysis system. Yellow or brownish-yellow staining represents positive staining.

Table 1 Comparison of blood gas and fluid volume among four groups (mean ± SD)

Group	pH value	MAP (mmHg)	Heart rate	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	Lactate (mmol/L)	Fluid volumes (mL)	Norepinephrine (μg/kg per minute)
A	7.38 ± 0.12	101 ± 8	240 ± 13	93.9 ± 2.23	38.65 ± 1.78	1.50 ± 0.34	0	0
B	6.89 ± 0.13[a]	37 ± 11[a]	65 ± 20[a]	50.19 ± 3.78[a]	34.54 ± 1.89	6.98 ± 2.45[a]	0	0
C	7.21 ± 0.15[c]	95 ± 10[c]	260 ± 15[c]	62.34 ± 2.46[c]	35.09 ± 2.01	4.37 ± 1.36[c]	12 ± 3[c]	3.5 ± 1.8[c]
D	7.32 ± 0.17[e]	97 ± 11	255 ± 12	88.98 ± 3.17[e]	36.49 ± 1.84	3.59 ± 1.53[c]	5 ± 2[e]	0.8 ± 0.3[e]

[a]$P < 0.05$ *vs* Group A; [c]$P < 0.05$ *vs* Group B; [e]$P < 0.05$ *vs* Group C. MAP: Mean arterial pressure.

Table 2 Comparison of wet/dry wight and redox indicator among four groups (mean ± SD)

Group	Wet/dry wight	Inhibition of •OH (U/mg protein)	MDA (nmol/mg protein)	MPO (U/g wet tissue)	SOD (U/mg protein)
A	3.85 ± 0.34	87.52 ± 3.45	2.98 ± 0.64	1.69 ± 0.14	365.78 ± 10.26
B	6.89 ± 0.23[a]	12.89 ± 1.52[a]	10.17 ± 1.12[a]	6.79 ± 1.02[a]	250.32 ± 8.56[a]
C	6.96 ± 0.25	13.14 ± 2.24	9.56 ± 1.37	6.02 ± 1.16	262.98 ± 5.47
D	4.62 ± 0.27[c]	66.31 ± 2.98[c]	4.45 ± 1.13[c]	2.58 ± 0.21[c]	331.15 ± 9.64[c]

[a]$P < 0.05$ *vs* Group A; [c]$P < 0.05$ *vs* Group C. •OH: Hydroxyl radical; MDA: Malonaldehyde; MPO: Mmyeloperoxidase; SOD: Superoxide dismutase.

Western blotting analysis

Levels of Fas and Bcl2 proteins in lung tissue were quantified by Western blotting as described previously with some minor modifications[12]. Briefly, about 10 mg frozen lung tissue were immersed in the tissue lysates at a ratio of 1:10 w/v, then submitted to ultrasound homogenation for 1 min followed by centrifugation at 13 000 rpm for 5 min at 4 ℃. The supernatant was collected, and total protein concentration was measured by the Bradford method. About 15 μg total proteins was loaded in each well and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 1 h. After running, the proteins were transferred onto the polyvinylidene difluoride membranes (Millipore, United States). The membranes were blocked with 5% fat-free milk, and then were incubated with Tris-buffered-daline Tween20-containing the primary antibodies (1:500 dilution) overnight at 4 ℃. After extensive washing, the second antibody was added, and the immunocomplexes were then detected using an enhanced chemiluminescence Western blotting detection kit (GE, Healthcare). The relative densities of the protein bands were analyzed using the Quantity One software from Bio-Rad. Data were represented in relative arbitrary units.

Statistical analysis

All the data analyses were carried out using the statistical analysis software SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, United States). Data were presented as the mean ± SD. Significant differences between the groups were analyzed by analysis of variance, and differences were considered significant at $P < 0.05$.

RESULTS

Fluid volume and blood gas parameters

Compared with Group A, the pH, MAP, heart rate and oxygen partial pressure of Group B were significantly decreased; however, a significant increase in lactate value was observed ($P < 0.05$). The pH, MAP, heart rate and oxygen partial pressure were increased obviously, and the lactate value was clearly decreased in Group C as compared with Group B. Furthermore, a significant increase in pH value and oxygen partial pressure and decrease in lactate value were also observed in Group D compared with Group C ($P < 0.05$). There was no significant difference in MAP and heart rate between Group D and Group C. Total fluid volume and the amount of norepinephrine used in Group D were significantly less than in Group C ($P < 0.05$). There was no significant difference in partial pressure of carbon dioxide among the four groups ($P > 0.05$; Table 1).

OH, MDA, MPO and SOD levels and lung W/D values

In Group B, the levels of OH, MDA, and MPO were significantly increased, and the level of SOD was significantly decreased compared with Group A ($P < 0.05$). Compared with Group B, lower levels of OH, MDA and MPO, and a higher level of SOD in Group C were also observed, but there was no significant difference between the two groups ($P > 0.05$). However, compared with Group C, there were also significant decreases in the levels of OH, MDA and MPO, and a significant increase in the SOD level in Group D ($P < 0.05$). The lung W/D value in Group B was significantly increased compared with group A ($P > 0.05$), and a higher lung W/D value was observed in Group C compared with Group B, but there was no significant difference between the two groups ($P > 0.05$). However, there was a significant decrease in the lung W/D value in Group D compared with Group C ($P < 0.05$; Table 2).

HE staining of lung tissues

HE-stained lung tissue sections were observed under

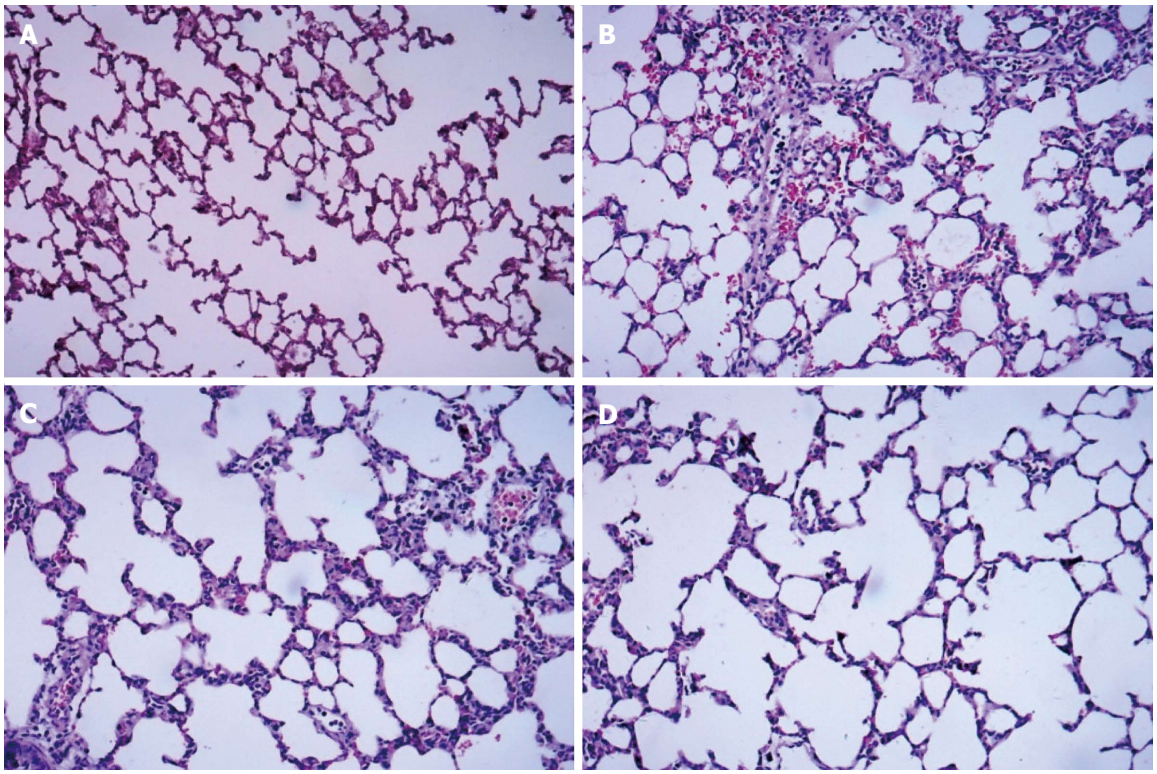

Figure 1 Hematoxylin and eosin staining of dorsal lobe of right lung tissue. A: Control group; B: Septic shock group; C: The early fluid resuscitation-treated group; D: Combined early fluid resuscitation and 2% hydrogen inhalation-treated group. Lung tissues were fixed with 4% paraformaldehyde for more than 24 h. After dehydrating and embedding, they were cut into 5 μm slices, and were stained with hematoxylin and eosin. The pathogenesis of lung injury was observed under a microscope (original magnification × 200). Normal alveolar structure was found in Group A. In Group B, disorders of the alveolar structures, severe neutrophil infiltration in the alveoli and alveolar capillary congestion were observed. In Group C, the extent of neutrophil accumulation and the alveolar-capillary exudate were reduced compared with Group B. However, significant decreases in alveolar damage were found in Group D, and there was significant improvement in alveolar edema compared with Group C.

Table 3 Level of inflammatory mediators in the bronchial alveolar lavage fluid of four groups (mean ± SD)

Group	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF-α (pg/mL)
A	35.88 ± 3.12	24.29 ± 4.23	19.65 ± 1.78
B	446.89 ± 37.24[a]	411.19 ± 32.78[a]	158.54 ± 16.89[a]
C	387.79 ± 31.58[c]	347.34 ± 29.37[c]	104.09 ± 9.18[c]
D	118.32 ± 11.38[e]	96.45 ± 10.25[e]	47.58 ± 3.65[e]

[a]$P < 0.05$ *vs* Group A; [c]$P < 0.05$ *vs* Group B; [e]$P < 0.05$ *vs* Group C. TNF-α: Tumor necrosis factor-α; IL: Interleukin.

a light microscope. The normal alveolar structure was found in Group A, and no hyperemia, neutrophil infiltration, and interstitial edema in the interstitium were observed (Figure 1A). In Group B, disorders of the alveolar structures, the collapse of alveoli, loss of integrity of the alveolar wall, severe neutrophil infiltration in the alveoli, and alveolar capillary congestion were observed, and the alveolar walls were thickened by edema (Figure 1B). In Group C, the extent of neutrophil accumulation and the alveolar-capillary exudate were reduced compared with Group B, but there was no significant improvement in alveolar edema (Figure 1C). Compared with Group B, significant decreases in alveolar damage were found in Group D (Figure 1D), and there was significant improvement in alveolar edema compared with Group C.

Cytokine profiles in lung tissues

Compared with Group A, significant increases in levels of IL-6, IL-8 and TNF-α were found in Group B ($P < 0.05$). The expression of IL-6, IL-8 and TNF-α was significantly decreased in Group C as compared with Group B. In addition, the expression of IL-6, IL-8 and TNF-α was even lower in Group D than in Group C, and the difference was significant (Table 3).

Expression of Fas and Bcl2-immunoreactivity in lung tissues

The positive expression of Fas and Bcl2 protein in lung tissue was demonstrated by the presence of brown-yellow granules, mostly located in the nucleolus cytoplasm (Figure 2). Most bronchial epithelial cells, alveolar epithelial cells and inflammatory cells were Fas-positive and Bcl-positive cells, and Fas and Bcl2 were expressed at low levels in normal lung tissue (Figure 2A and E). The expression of Fas proteins was up-regulated, while the expression of Bcl2 proteins was down-regulated in Group B (Figure 2B and F) compared with Group A. In Group C, however, the expression of Fas proteins was down-regulated, and the expression of Bcl2 proteins was up-regulated (Figure 2C and G) compared with Group B ($P < 0.05$). In addition, the lower expression of Fas proteins and the higher expression of Bcl2 proteins were

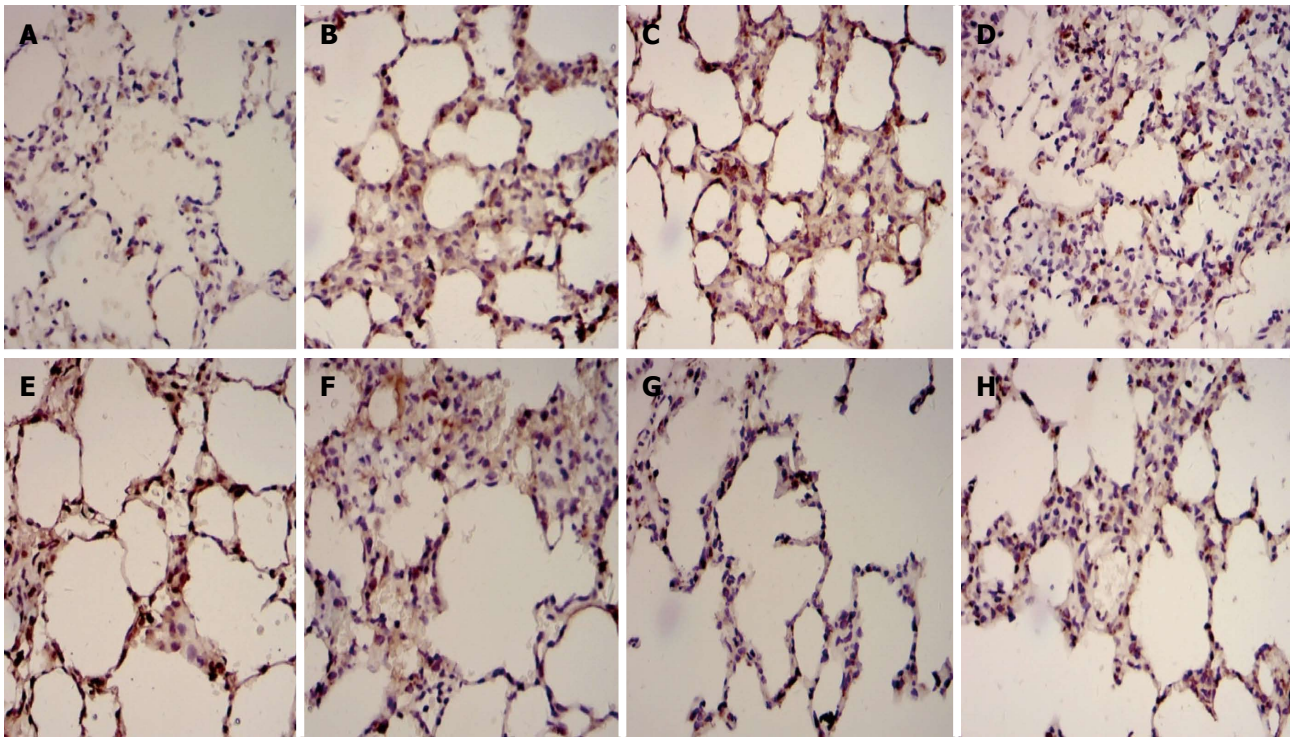

Figure 2 Immunohistochemical examples for Fas and Bcl2 in dorsal lobe of right lung tissue. A: Fas in control group; B: Fas in septic shock group; C: Fas in early fluid resuscitation-treated group; D: Fas in early fluid resuscitation + 2% hydrogen inhalation-treated group; E: Bcl2 in control group; F: Bcl2 in septic shock group; G: Bcl2 in early fluid resuscitation-treated group; H: Bcl2 in early fluid resuscitation + 2% hydrogen inhalation-treated group. Examples were examined under a light microscope equipped with an image analysis system. Yellow or brownish-yellow staining represents positive staining. The expression of Fas proteins was up-regulated, while the expression of Bcl2 proteins was down-regulated in Group B compared with Group A. In Group C, however, the expression of Fas proteins was down-regulated, and the expression of Bcl2 proteins was up-regulated compared with Group B. In addition, the lower expression of Fas proteins and the higher expression of Bcl2 proteins were observed in Group D compared with Group C.

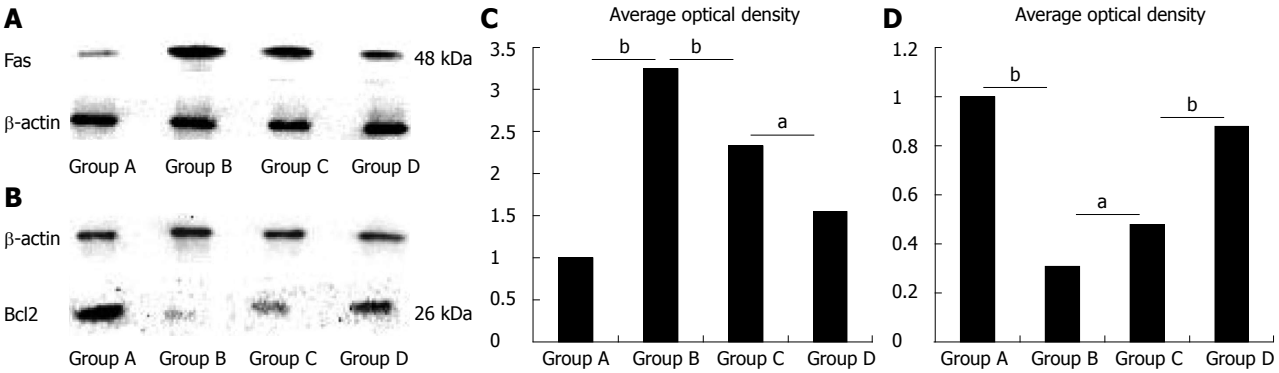

Figure 3 Levels of Fas and Bcl2 proteins in dorsal lobe of right lung tissues quantified by Western blotting. β-actin was used as a loading control. The relative Fas (A) and Bcl2 (B) protein levels of lung tissues from the four groups were expressed as arbitrary units after normalization with β-actin (C: Fas; D: Bcl2). Data were considered significant at $^{a}P < 0.05$ and highly significant at $^{b}P < 0.01$ *vs* the control group. The level of Fas protein was up-regulated, while the level of Bcl2 protein was down-regulated in Group B *vs* Group A. However, in Group C the level of Fas proteins was down-regulated, and the level of Bcl2 proteins was up-regulated *vs* Group B ($P < 0.01$). In addition, the lower level of Fas protein and the higher level of Bcl2 protein was found in Group D compared with Group C ($P < 0.05$).

observed in Group D (Figure 2D and H) compared with Group C ($P < 0.05$; Table 4).

Levels of Fas and Bcl2 protein in lung tissues

The level of Fas protein was up-regulated, while the level of Bcl2 protein was down-regulated in Group B compared with Group A ($P < 0.01$). However, in Group C, level of Fas protein was down-regulated, and the level of Bcl2 protein was up-regulated compared with Group B ($P < 0.01$; Figure 3). In addition, a lower level of Fas protein and a higher level of Bcl2 protein were found in Group D compared with Group C ($P < 0.05$; Figure 3).

Levels of DAO in intestinal tissues

The level of DAO in Group B was significantly increased compared with Group A ($P < 0.05$), and the level of DAO was even higher in Group C ($P < 0.05$). But the level of DAO in Group D was significantly decreased

Table 4 Comparison of average optical density and the positive area ratio of Fas and Bcl2 among the four groups (n = 15, mean ± SD)

Group	Average optical density (10^{-3})		Positive area ratio (%)	
	Fas	Bcl2	Fas	Bcl2
A	0.802 ± 0.221	1.260 ± 0.128	2.737 ± 0.881	11.012 ± 0.792
B	1.549 ± 0.343[a]	0.638 ± 0.072[a]	6.441 ± 0.331[a]	4.121 ± 0.313[a]
C	1.329 ± 0.311[c]	1.103 ± 0.099[c]	4.876 ± 0.887[c]	4.376 ± 0.250[c]
D	1.255 ± 0.400[e]	1.141 ± 0.043[e]	3.247 ± 0.621[e]	10.432 ± 0.791[e]

[a]P < 0.05 *vs* Group A; [c]P < 0.05 *vs* Group B; [e]P < 0.05 *vs* Group C.

Table 5 Levels of serum diamine oxidase and inflammatory mediators in the bronchial alveolar lavage fluid of four groups (mean ± SD)

Group	DAO (kU/L)	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF-α (pg/mL)
A	4.32 ± 0.33	50.38 ± 3.24	35.93 ± 4.37	25.32 ± 2.18
B	6.54 ± 0.68[a]	478.33 ± 30.78[a]	458.19 ± 31.32[a]	167.41 ± 14.47[a]
C	6.89 ± 0.77[c]	394.21 ± 28.15[c]	395.34 ± 28.46[c]	121.05 ± 8.01[c]
D	5.14 ± 0.44[c,e]	145.26 ± 12.33[e]	123.12 ± 10.23[e]	36.27 ± 3.54[e]

[a]P < 0.05 *vs* Group A; [c]P < 0.05 *vs* Group B; [e]P < 0.05 *vs* Group C. TNF-α: Tumor necrosis factor-α; IL: Interleukin; DAO: Diamine oxidase.

Table 6 Chiu's score and levels of malonaldehyd and myeloperoxidase of small intestine (mean ± SD)

Group	Chiu's score	MDA (nmol/mg protein)	MPO (U/g wet tissue)
A	0.37 ± 0.33	2.09 ± 0.33	1.55 ± 0.12
B	3.30 ± 0.89[a]	6.19 ± 1.38[a]	6.54 ± 0.99[a]
C	3.79 ± 1.12[c]	6.89 ± 1.26[c]	7.05 ± 1.01[c]
D	2.12 ± 0.67[c,e]	2.98 ± 0.45[c,e]	2.32 ± 0.34[c,e]

[a]P < 0.05 *vs* Group A; [c]P < 0.05 *vs* Group B; [e]P < 0.05 *vs* Group C. MDA: Malonaldehyd; MPO: Myeloperoxidase.

compared with both Group B and Group C (P < 0.05; Table 5).

HE staining of small intestinal tissues

In Group A, the structure of the small intestinal mucosa was intact, and normal intestinal mucosa was observed (Figure 4A). In Group B, glands of the small intestine were significantly damaged, and severe edema of mucosal villi, neutrophil infiltration, epithelial cell sloughing off, and structural changes in the epithelium of the small intestine and even small bowel ulceration were observed (Figure 4B). The damage mentioned above was far more severe in Group C (Figure 4C). But in Group D, the damage was significantly reduced (Figure 4D). The Chiu's score of Group B was significantly higher than Group A (P < 0.05), and the Chiu's score of Group C was even higher compared with Group B. But the Chiu's score of Group D was significantly decreased compared with Group B and Group C (Table 6).

Transmission electron microscopic analysis of small intestinal samples

In Group A, normal microvilli on the surface of epithelial cells of the intestine were observed. The mitochondria, lysosome and rough endoplasmic reticulum maintained normal morphology (Figure 5A). In Group B, disarrangement of the epithelial surface and intestinal microvillus reduction were observed. Mitochondria present in the cytoplasm also showed vacuolization, and the cristae of mitochondria were reduced. Heterochromatin nuclei showed margination phenomena, membrane rupture, and widened nuclear gap (Figure 5B). The microvilli on the surface of epithelial cells of the intestine were sparse in Group C; there was obvious reduction of the cristae of mitochondria, severe vacuolization of mitochondria and an abundance of marginated heterochromatin nuclei. The amount of rough endoplasmic reticulum was reduced, and more severe rough endoplasmic reticulum swelling and expansion were observed (Figure 5C). In Group D, the microvilli were missing to a small extent, and the heterochromatin nuclei showed only mild margination (Figure 5D).

Levels of MDA and SOD in small intestinal tissues

The levels of MDA and SOD in Group B were significantly increased compared with Group A (P < 0.05). Levels of MDA and SOD were higher in Group C than in Group B. In Group D, MDA and SOD were significantly decreased compared with both Group B and Group C (Table 6).

Cytokine profiles in small intestinal tissues

Levels of IL-6, IL-8 and TNF-α were significantly increased in Group B compared with Group A (P < 0.05). The expression of IL-6, IL-8 and TNF-α was significantly lower in Group C than in group B. In addition, the expression of IL-6, IL-8 and TNF-α in Group D was even lower compared with Group C, and the difference was significant (P < 0.05; Table 5).

DISCUSSION

In the present study, we investigated the effects of combined early fluid resuscitation and 2% hydrogen on the lung and small intestine in a LPS-induced septic shock rat model. Our study showed that early fluid resuscitation and inhalation of 2% hydrogen could decrease the oxidative damage and inhibit the over-expression of inflammatory factors (IL-6, IL-8 and TNF-α) in the lung tissue of the septic shock rats. This treatment could also down-regulate the expression of proapoptotic protein Fas, and up-regulate the expression of anti-apoptotic protein Bcl2 in the lung tissue. These data suggest that the application of combined early fluid resuscitation and inhalation of 2% hydrogen may protect the lung from septic shock damage by inhibiting the inflammatory reaction and apoptosis in comparison with traditional fluid resuscitation alone. Early fluid resuscitation and inhalation of 2% hydrogen not only decreased the oxidative damage and

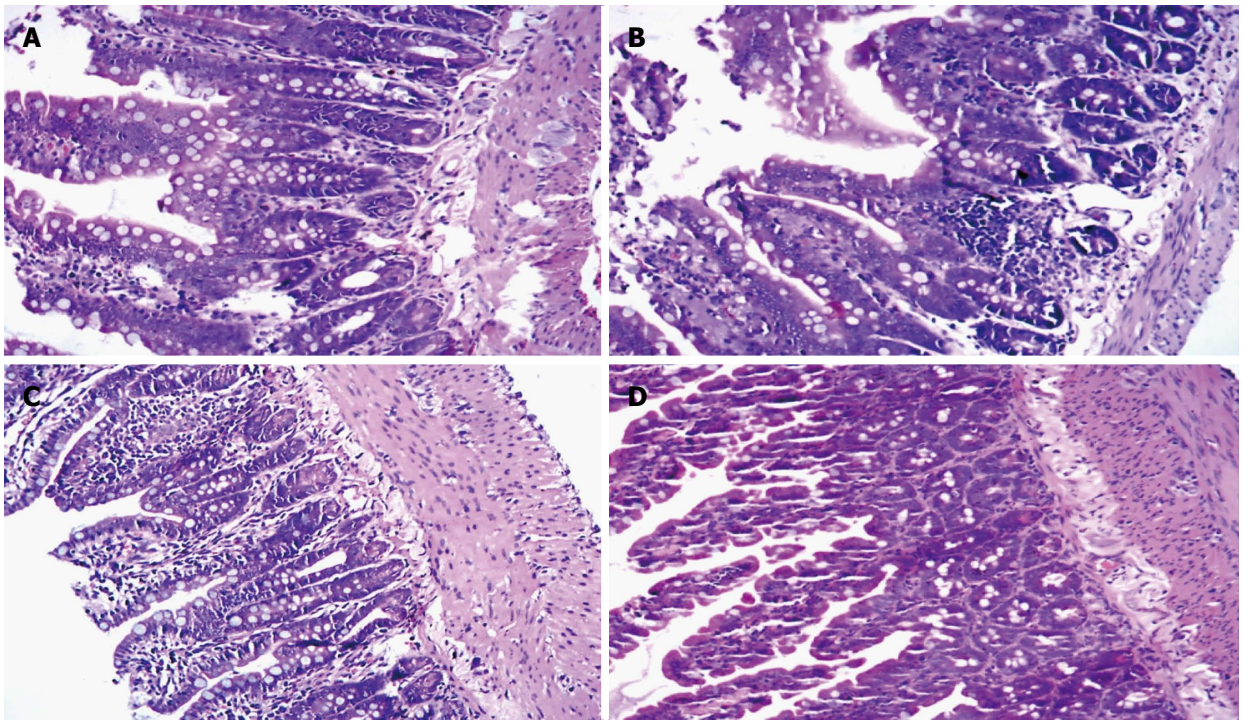

Figure 4 Hematoxylin and eosin staining of small intestine tissue. A: Control group; B: Septic shock group; C: Early fluid resuscitation-treated group; D: Early fluid resuscitation + 2% hydrogen inhalation-treated group. Small intestinal tissues were fixed with 4% paraformaldehyde for more than 24 h. After dehydrating and embedding, they were cut into 5 μm slices, and were stained with hematoxylin and eosin. The pathogenesis of small intestine injury was observed under a light microscope (original magnification × 200). In Group A, normal intestinal mucosa was observed. In Group B, glands of small intestine were significantly damaged, and severe edema of mucosal villi, neutrophil infiltration, epithelial cell sloughing off, and even small bowel ulceration were observed. The damage mentioned above was far more severe in Group C. But in Group D, the damage was significantly reduced.

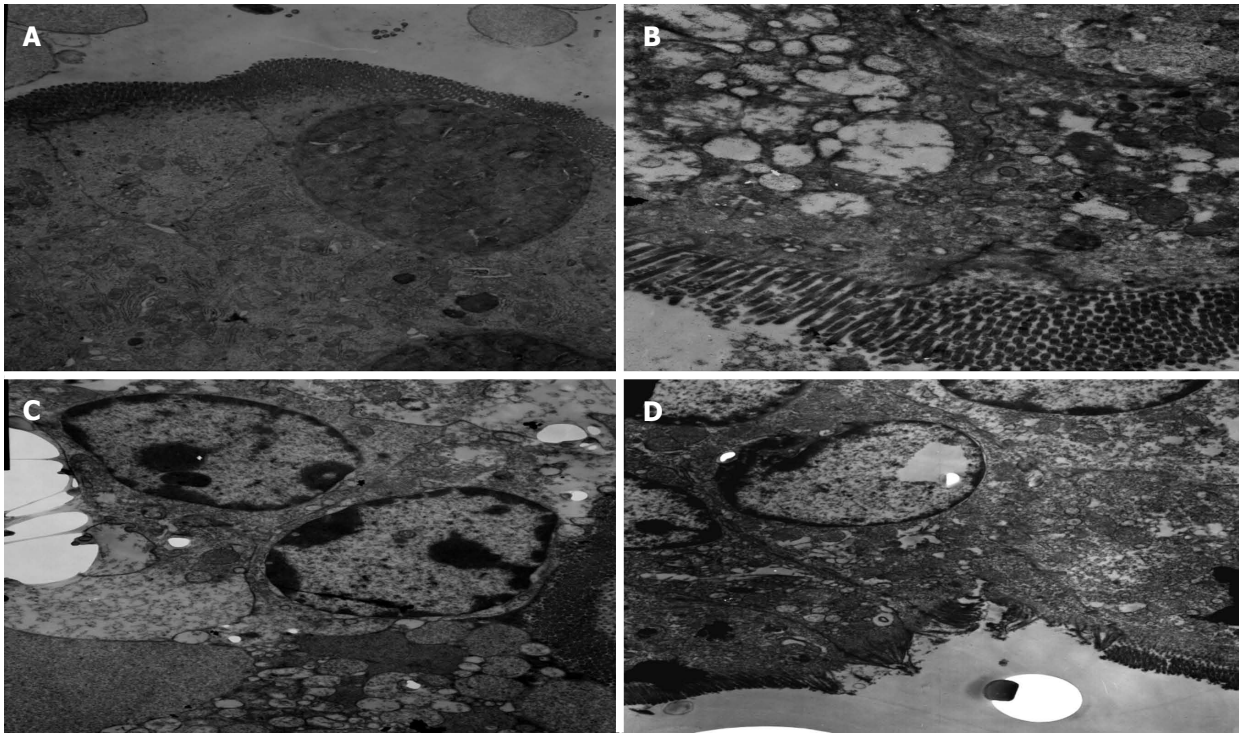

Figure 5 Changes in the organelles of small intestinal tissues under transmission electron microscope. A: Control group; B: Septic shock group; C: Early fluid resuscitation-treated group; D: Early fluid resuscitation + 2% hydrogen inhalation-treated group. The changes in the organelles were observed under transmission electron microscope. In Group A, normal microvilli on the surface of epithelial cells of the intestine were observed. In Group B, intestinal microvillus reduction was observed. Mitochondria showed vacuolization and heterochromatin nuclei showed margination phenomena. The microvilli on the surface of epithelial cells of the intestine were sparse in Group C, and reduction of the cristae of mitochondria, an abundance of marginated heterochromatin nuclei, and severe rough endoplasmic reticulum swelling and expansion were observed. In Group D, the microvilli were missing to a small extent, and the heterochromatin nuclei showed only mild margination.

the levels of IL-6, IL-8 and TNF-α in plasma, but also protected the intestinal mucosa from mechanical injury.

Effects of combined early fluid resuscitation and hydrogen inhalation on lung injury in a LPS-induced septic shock rat model

Previous studies have indicated that MPO is a peroxidase enzyme released by activated polymorphonuclear neutrophils, which can be used as an indicator of the level of tissue neutrophils. MDA is one of the toxic metabolites of ROS induced lipid peroxidation, and SOD is one of the most important free radical scavenging enzymes. Levels of MDA and SOD can reflect the extent of oxidative stress. In the present study, the levels of MDA, SOD and MPO were used as an indicator for oxidative stress[13-15]. The results showed that the levels of MDA and MPO were significantly increased, however, the level of SOD was significantly decreased in the septic shock rats, and severe neutrophil infiltrates were observed in the alveoli. These data suggest that changes in the lung oxidant-antioxidant status may be associated with lung injury in septic shock rats. There was no obvious improvement in oxidative stress in the traditional fluid resuscitation-treated group compared with the septic shock group, but there was significant improvement in the indicators above in the early fluid resuscitation and inhalation of 2% hydrogen treatment group. Early fluid resuscitation and inhalation of 2% hydrogen attenuated septic shock-induced organ injury, and decreased neutrophil infiltrate in the alveoli. Significant improvements in alveolar edema and reduced alveolar damage were also observed. Previous studies have indicated that hydrogen, as a selective antioxidant with a therapeutic antioxidant activity, can easily pass through the cell membrane and reach the organelles[16,17]. Hydrogen can effectively protect against organ damage induced by selectively clearing •OH. These findings indicate that 2% hydrogen may selectively reduce •OH and provide a beneficial effect on septic shock.

Amplification of inflammatory responses and cell apoptosis also play an important role in the pathogenesis of sepsis-induced acute lung injury (ALI)[18,19]. In the present study, the levels of IL-6, IL-8 and TNF-α in BALF were significantly increased in the LPS-induced ALI rats compared with the control group. Our data showed that the early fluid resuscitation and inhalation of 2% hydrogen treatment reduced the levels of IL-6, IL-8 and TNF-α in BALF in comparison with the traditional fluid resuscitation-treated group, and these results are consistent with the previous study[1]. In the process of sepsis-induced ALI, the expression of Fas protein in the lung epithelial cell was up-regulated, and blocking FAS-mediated apoptosis could reduce the LSP-induced lung injury[20]. As one of the most important anti-apoptotic factors, Bcl-2 plays an important role in regulating the progress of apoptosis, and it is considered as a cytoprotective factor[21]. In the present study, decreases in lung cell apoptosis were observed in the early fluid resuscitation-treated group, and further decreases in lung cell apoptosis were observed in the early fluid resuscitation and inhalation of 2% hydrogen treated group. These results demonstrated that co-treatment could down-regulate the levels of Fas protein and up-regulate the levels of Bcl2 protein, which may inhibit ALI by inducing apoptosis, and may protect lung function.

Effects of combined early fluid resuscitation and inhalation of 2% hydrogen on small intestine in a LPS-induced septic shock rat model

Clinical observations and animal experiments have shown that the gastrointestinal tract is one of the most important target organs during septic shock. In early shock, blood flow in the intestinal wall is significantly decreased because of the redistribution of blood, and intestinal mucosal barrier dysfunction is induced by hypoxia and ischemia. Previous studies show that intestinal mucosal edema and bacterial translocation are closely related to the occurrence of MODS[22]. In the present study, bowel mucosal structural changes were observed in the septic shock rats, including significant damage of the small intestine, severe edema of mucosal villi, sloughing off of epithelial cells, structural changes in the epithelium of the small intestine and even small bowel ulceration. Serum DAO activity, which was used as a marker of intestinal integrity, was significantly increased[23]. All these indicators suggest that the intestinal mucosal mechanical barrier is severely damaged during septic shock. Early fluid resuscitation treatment alone was not effective in reducing pathological changes of the small intestine; on the contrary, pathological changes were more severe, including more severe bowel wall edema, higher Chiu's score, and higher level of serum DAO activity in the early fluid resuscitation-treated group compared with the septic shock group. But 2% hydrogen gas treatment significantly reduced the pathological changes of the small intestine, indicating that early fluid resuscitation and inhalation of 2% hydrogen could protect the intestinal mucosal mechanical barrier, inhibit bacterial translocation, and then effectively prevent enterogenous sepsis, and protect the function of other organs in the body[24,25].

Increased endothelial cell ischemia and hypoxia were observed during septic shock. The endothelial cells could release large amounts of cytokine and chemokine, and induce large amounts of neutrophil aggregation. During the removal of harmful pathogens, large amounts of oxygen free radical, which increase the intestinal damage, were released[26]. After fluid resuscitation treatment, large amounts of oxygen free radical were generated in the course of ischemia and reperfusion injury. Thus, oxygen free radicals were considered as one of the major causes of intestinal damage during septic shock. Our data indicate that oxygen free radicals were involved in the severe intestinal mucosal damage, and pathological changes were more severe after fluid resuscitation treatment. Early fluid resuscitation and inhalation of 2% hydrogen could significantly decrease the level of MDA and MPO,

and mitigate the oxidative damage, indicating that 2% hydrogen may selectively reduce •OH, reducing ischemia/reperfusion-induced organ damage.

Intestinal mucosal injury can lead to the release of inflammatory mediators which play an important role in the process of shock-induced intestinal tissue injury[27]. The inflammatory mediators were one of the initiation factors of MODS, and could worsen organ dysfunction[28]. During the shock period, the intestinal tract is the main organ to produce TNF-α[29]. Release of TNF-α increases chemotaxis, aggregation and adhesion to endothelial cells of neutrophils. Then more inflammatory mediators such as IL-6 and IL-8 are also released, leading to aggravated intestinal mucosal injury[30]. In the present study, the levels of TNF-α, IL-6 and IL-8 in serum were significantly increased in septic shock rats, but the levels of TNF-α, IL-6 and IL-8 in serum were decreased after early fluid resuscitation treatment, while early fluid resuscitation and inhalation of 2% hydrogen gas further decreased the levels of TNF-α, IL-6 and IL-8 in serum compared with early fluid resuscitation only. These data demonstrated that the association of early fluid resuscitation and inhalation of 2% hydrogen gas may protect small intestine function by reducing inflammatory mediators.

In summary, combined early fluid resuscitation and inhalation of 2% hydrogen is beneficial for septic shock and septic shock-associated lung or small intestine injury, which is associated with a decrease of oxidative stress and inhibition of the inflammatory response. We conclude that combined early fluid resuscitation and inhalation of 2% hydrogen may be an effective therapeutic strategy for ALI and MODS in patients with septic shock.

COMMENTS

Background

Sepsis shock is a common complication of infection, severe trauma, and major operation in patients. However, the effective therapy method needs to be developed.

Research frontiers

Early fluid resuscitation has been demonstrated to have some protective effects on the sepsis shock -induced acute lung injury, but the effective is not significant. Recent studies indicate that hydrogen inhalation can significantly decrease the oxidative stress in septic animal model. The objective of this study is to explore the effects of combined early fluid resuscitation and hydrogen inhalation on septic shock induced lung and intestine injuries.

Innovations and breakthroughs

This is the first study to report that combined early fluid resuscitation and hydrogen inhalation exerts a potency to effectively attenuate lung and intestine injuries.

Applications

The paper provides the experiment basis for the clinical application of early fluid resuscitation and hydrogen inhalation to prevent sepsis induced lung and intestine injury.

Terminology

Sepsis shock is a common complication of infection, severe trauma, and major operation in patients. Sepsis shock leads to severe, hypoxia, and reperfusion injury of lung and intestine ischemia. The inflammatory factors in these tissues will be activated and may consequently develop the multiple organ dysfunction syndrome, which is associated with high morbidity and mortality rates. Early fluid resuscitation and hydrogen have the potential to clear these inflammatory factors.

Peer review

This study shows that combined early fluid resuscitation and hydrogen inhalation can significantly decrease septic shock induced lung and intestine injuries compared with early fluid resuscitation alone. The study is innovative and with potential therapeutic interest.

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BRIEF ARTICLE

Development and validation of a registry-based definition of eosinophilic esophagitis in Denmark

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Abstract

AIM: To develop and validate a case definition of eosinophilic esophagitis (EoE) in the linked Danish health registries.

METHODS: For case definition development, we queried the Danish medical registries from 2006-2007 to identify candidate cases of EoE in Northern Denmark. All International Classification of Diseases-10 (ICD-10) and prescription codes were obtained, and archived pathology slides were obtained and re-reviewed to determine case status. We used an iterative process to select inclusion/exclusion codes, refine the case definition, and optimize sensitivity and specificity. We then re-queried the registries from 2008-2009 to yield a validation set. The case definition algorithm was applied, and sensitivity and specificity were calculated.

RESULTS: Of the 51 and 49 candidate cases identified in both the development and validation sets, 21 and 24 had EoE, respectively. Characteristics of EoE cases in the development set [mean age 35 years; 76% male; 86% dysphagia; 103 eosinophils per high-power field (eos/hpf)] were similar to those in the validation set (mean age 42 years; 83% male; 67% dysphagia; 77 eos/hpf). Re-review of archived slides confirmed that the pathology coding for esophageal eosinophilia was correct in greater than 90% of cases. Two registry-based case algorithms based on pathology, ICD-10, and pharmacy codes were successfully generated in the development set, one that was sensitive (90%) and one that was specific (97%). When these algorithms were applied to the validation set, they remained sensitive (88%) and specific (96%).

CONCLUSION: Two registry-based definitions, one highly sensitive and one highly specific, were developed and validated for the linked Danish national health databases, making future population-based studies feasible.

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Key words: Eosinophilic esophagitis; Denmark; Epidemiology; Case definition; Sensitivity; Specificity

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INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic immune-mediated disorder characterized by symptoms of esophageal dysfunction and infiltration of the esophageal mucosa by eosinophils[1]. While the first case was described in 1978[2], a set of case series in the early 1990s highlighted what are now viewed as the classic clinicopathologic features of EoE[3-5]. Since that time, there has been a remarkable increase in the incidence and prevalence of EoE[6-14], EoE has been detected in up to 16% of patients undergoing upper endoscopy for dysphagia[15-17], and EoE is now the leading cause of food impaction in patients presenting to emergency departments[18-20]. Most data regarding the evolving epidemiology of EoE originate from tertiary centers[7-13], and there have been few population-based studies of EoE across centers or at a national level.

The national population-based medical registries of Denmark, which link medical records, diagnostic codes, pathology findings, and prescription medications *via* an individual identifier assigned to every person in the country[21-24], offer a unique opportunity to systematically study the epidemiology of EoE. However, no validated registry-based case definitions of EoE exist, and this limitation hampers any attempt to accurately study EoE in Denmark and other countries where national health databases are maintained.

The aims of this study were to characterize candidate and definite EoE cases in Northern Denmark detected through a search of the health registries, and to develop and validate a registry-based case definition of EoE in the linked Danish clinical, pathology, and pharmacy databases that could be used for future epidemiologic study.

MATERIALS AND METHODS

Denmark health registries

This retrospective study of the Danish medical registries was approved by both the University of North Carolina IRB and the Danish Data Protection Agency (record number 2010-41-4986). Denmark, with its stable population of approximately 5.5 million people, is well-suited for epidemiological studies[21-24]. The establishment of the Civil Registration System in 1968 allows information about the same person to be linked across independent registries by using the Civilian Registration Number, a unique identifier assigned to every person in the country[23].

Three of the comprehensive national medical registries in Denmark were utilized to generate the necessary data for this study. These included: the Danish National Registry of Patients, which houses International Classification of Diseases-10 (ICD-10) codes dating from 1994, hospital admission and discharge codes, surgical procedure codes, and outpatient visit codes[25]; the National Pathology Registry, which contains Systematized Nomenclature of Medicine (SNOMED) codes for pathologic specimens dating from 1997[24]; and the Aarhus University Prescription Database, which has outpatient prescription data for Northern Denmark using Anatomical Therapeutic Chemical (ATC) classification system codes[26].

For the purposes of this study we limited our analysis to Northern Denmark. This geographic area contains 1.8 million inhabitants, approximately 1/3 of the Danish population, and allowed ready access to the required pathology specimens for case verification, as described below.

Case definition development

This study utilized two independent sets of patients, one set in which to develop the case definition and one set in which to validate it.

For case definition development, we queried the National Pathology Registry from 2006-2007 in Northern Denmark to identify candidate cases of EoE. These were patients with esophageal eosinophilia as defined by the combination of SNOMED codes for esophageal biopsies (T62xxx) and tissue eosinophilia (M47150). These codes were assigned by the clinical pathologist at the time of specimen examination and interpretation. Next, all ICD-10 diagnostic and ATC prescription codes for these patients were obtained from the National Registry of Patients and the Aarhus University Prescription Database, respectively. Finally, the original archived pathology slides were obtained and, using a previously validated protocol[27], were re-reviewed by the study pathologist (Vyberg M) to determine the maximum eosinophil count [eosinophils per high-power field (eos/hpf); hpf = 0.24 mm^2].

From this pool of subjects with esophageal eosinophilia, we identified the subset of patients with a confirmed diagnosis of EoE as per consensus diagnostic guidelines[1,28]. Specifically, these patients had symptoms of esophageal dysfunction, a maximum eosinophil count $\geqslant$ 15 eos/hpf on pathology re-review, and no other competing causes of esophageal eosinophilia identified. While the current consensus guidelines allow reflux and EoE to overlap[1], we attempted to minimize this overlap by ensuring that there was not a mixed inflammatory infiltrate on the re-reviewed esophageal biopsies and by assessing use of anti-acid mediations in the months preceding the diagnostic endoscopy (see below). These subjects with confirmed EoE comprised the reference standard for the case definition development set.

We used an iterative process to develop a case definition of EoE based on previously described methodology for gastrointestinal disorders used in the Danish and other administrative databases[29-31]. First, we empirically selected a combination of SNOMED, ICD-10 and ATC codes that were potentially pertinent to the diagnosis of EoE and could exclude disorders on the differential diagnosis for esophageal eosinophilia as well as patients with non-EoE conditions with similar symptom profiles. Next, the National Pathology Registry, National Registry of Pa-

Table 1 Characteristics of the case definition development set (2006-2007) and validation set (2008-2009) *n* (%)

	Development set (2006-2007)				Validation set (2007-2008)			
	Overall (*n* = 51)	EoE cases (*n* = 21)	Sensitive definition (*n* = 31)	Specific definition (*n* = 9)	Overall (*n* = 49)	EoE cases (*n* = 24)	Sensitive definition (*n* = 26)	Specific definition (*n* = 6)
Age, yr (mean ± SD)	42 ± 19	35 ± 16	40 ± 18	38 ± 19	48 ± 21	42 ± 15	49 ± 19	49 ± 19
Male	38 (75)	16 (76)	22 (71)	7 (78)	36 (73)	20 (83)	21 (81)	4 (67)
Dysphagia	24 (47)	18 (86)	28 (90)	8 (89)	19 (39)	16 (67)	18 (69)	3 (50)
Esophageal foreign body	15 (29)	11 (52)	14 (45)	3 (33)	8 (16)	2 (8)	4 (15)	1 (17)
Rhinitis/sinusitis	5 (10)	2 (10)	2 (6)	1 (11)	0 (0)	0 (0)	0 (0)	0 (0)
Asthma	3 (6)	1 (5)	1 (3)	0 (0)	2 (4)	2 (8)	2 (8)	1 (17)
PPI use	38 (75)	17 (81)	21 (68)	9 (100)	33 (67)	12 (50)	21 (81)	6 (100)
Maximum eosinophil count, eos/hpf (mean ± SD)	71 ± 53	103 ± 54	84 ± 58	93 ± 83	68 ± 71	77 ± 62	60 ± 35	43 ± 31

EoE: Eosinophilic esophagitis; PPI: Proton-pump inhibitor; eos/hpf: Eosinophils per high-power field.

tients, and Aarhus University Prescription Database were queried using this combination of codes to provide a set of possible EoE patients. Then, using the confirmed EoE cases previously identified as the reference standard, the sensitivity and specificity of this initial search strategy were determined within the population of patients with esophageal eosinophilia. Sensitivity was calculated by dividing the number of true positive EoE cases identified using the case definition by the total number of reference standard EoE cases. Specificity was calculated by dividing the number of true negative subjects identified using the case definition by the total number of subjects without EoE. By examining the coding and patient characteristics of those classified as false positive and false negative, the administration case definition was further refined, the databases were re-queried with the updated definition, and new operating characteristics were calculated. This process continued until sensitivity and specificity were optimized.

Case definition validation

Using the same methodology as described for case definition development, the case definition was validated in an independent population in Northern Denmark from 2008-2009. In brief, candidate cases of EoE were identified based on SNOMED codes, and a reference standard set of patients with confirmed EoE in the new time frame was created after re-review of original pathology slides and all coding data. The administrative case definition algorithm was then applied to the National Pathology Registry, National Registry of Patients, and Aarhus University Prescription Database, the set of possible EoE cases was generated, and sensitivity and specificity were calculated using the confirmed EoE cases as the reference standard.

RESULTS

Characteristics of the development set

There were 51 patients with esophageal eosinophilia identified in Northern Denmark from 2006-2007, and 21 (41%) had a confirmed diagnosis of EoE (Table 1). This group comprised the reference standard for the development set. Patients with esophageal eosinophilia had a mean age of 42 years, 75% were male, and almost half had dysphagia. While the EoE patients had a similar proportion of males (76%), they were somewhat younger (35 years), and 86% had dysphagia. The maximum eosinophil count was 71 eos/hpf in all patients with esophageal eosinophilia, and 103 eos/hpf in patients with confirmed EoE. The pathology re-review of slides confirmed that the SNOMED coding for esophageal eosinophilia was correct in the vast majority of cases, with 90% meeting criteria for histologic EoE (≥ 15 eos/hpf) (Figure 1).

Characteristics of the validation set

There were 49 patients with esophageal eosinophilia identified in Northern Denmark from 2008-2009, and 24 (49%) had a confirmed diagnosis of EoE (Table 1). This group comprised the reference standard for the validation set. Patients with esophageal eosinophilia had a mean age of 48 years, 73% were male, and 39% had dysphagia. While the EoE patients had a similar proportion of males (83%), they were also somewhat younger (42 years) and 67% had dysphagia. The maximum eosinophil count was 68 eos/hpf in all patients with esophageal eosinophilia, and 77 eos/hpf in patients with confirmed EoE. Overall, the clinical characteristics of the EoE cases in the development and validation sets were similar, and pathology re-review in the validation set also confirmed correct SNOMED coding in 90% of cases (Figure 1).

EoE case definition algorithms and operating characteristics

The iterative methodology for case definition development and validation yielded two registry-based case diagnostic algorithms, one that was highly sensitive and one that was highly specific. The highly sensitive case definition maximized the number of cases of EoE that were identified in the database search, while the highly specific definition accepted a lower sensitivity (i.e., not identifying all EoE cases) in exchange for ensuring that all cases were true EoE cases (i.e., minimizing false positives).

The final case definition algorithms are presented in Table 2. The "sensitive" algorithm has three steps. The

Table 2 Case definition algorithm

For both the sensitive and the specific case definitions:
(1) Include all patients with systematized nomenclature of medicine M47150 (finding of tissue eosinophil) and T62xxx (esophageal biopsy)
(2) Exclude patients if they have one of the following International Classification of Diseases-10 codes occurring at any time:
B20.x - human immunodeficiency virus
C15.x - malignant neoplasm of esophagus
C16.0 - malignant neoplasm of cardia
C92.x - myeloid leukemia
C94.x - other leukemias of specialized cell type
C96.x - other and unspecified lymph/heme malignancies
K22.0 - achalasia
K50.x - Crohn's disease
K52.8 - eosinophilic gastritis/duodenitis
T28.1 - burn of esophagus
T28.6 - corrosion of esophagus
(3) Include patients if one of the following International Classification of Diseases-10 codes is present at or before the date of the procedure:
K22.2 - esophageal obstruction
K30.9 - dyspepsia
P92.x - feeding problems of the newborn
R07.x - pain in throat and chest
R10.x - abdominal pain
R11.x - nausea/vomiting
R12.x - heartburn
R13.x - dysphagia
T18.0 - foreign body in mouth
T18.1 - foreign body in esophagus
T18.2 - foreign body in stomach
T18.9 - foreign body in genitourinary tract unspecified
T98.0 - sequelae of foreign body entering through a natural orifice
Additional requirement for the specific case definition:
(4) Include patients if there is a proton-pump inhibitor prescription (A02BCxx) or H2RA prescription (A02BAxx) within 2 mo of esophageal biopsy date, up to and including 2 d prior to the esophagogastroduodenoscopy

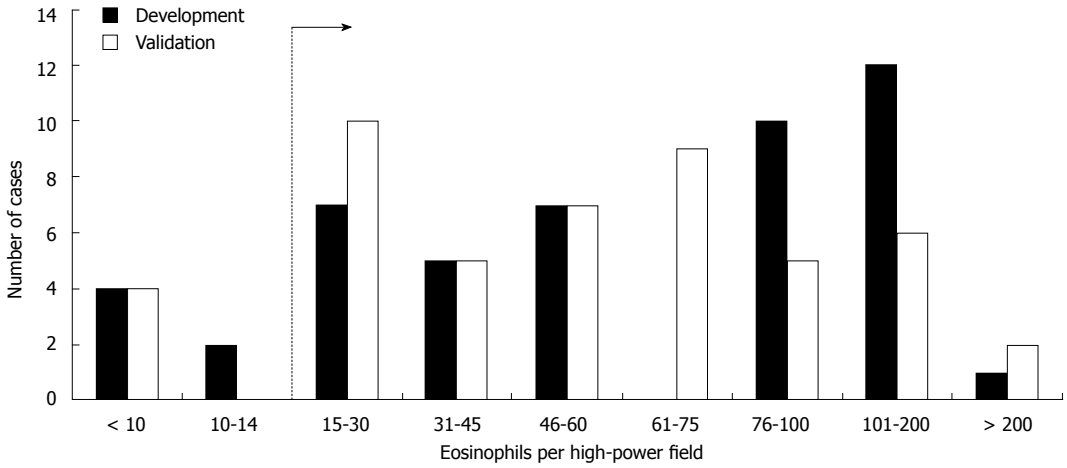

Figure 1 Distribution of levels of esophageal eosinophilia in the development and validation sets of candidate cases of eosinophilic esophagitis identified in the Denmark medical registries. Ranges of esophageal eosinophilia [in eosinophils per high-power field (eos/hpf)] determined after pathology re-review of archived slides are on the x axis. The typical histologic threshold for considering a diagnosis of eosinophilic esophagitis in the appropriate clinical context of 15 eos/hpf is indicated with a dotted line.

first uses SNOMED codes to include all patients with esophageal eosinophilia. The second excludes patients if they have any one of eleven ICD-10 codes that could cause non-EoE related esophageal eosinophilia or esophageal injury that could mimic EoE clinically. The third requires patients to have at least one of 13 ICD-10 codes for symptoms of esophageal dysfunction. The "specific" algorithm has an additional step to exclude false positives which requires a documented proton-pump inhibitor (PPI) or histamine-2 receptor antagonist prescription within 2 mo of the esophageal biopsy date.

When these algorithms were applied to the development set, the clinical characteristics of the cases identified for both the sensitive definition (n = 31; mean age 40 years; 71% male; 90% dysphagia; 84 eos/hpf) and the specific definition (n = 8; mean age 38 years; 78% male; 89% dysphagia; 93 eos/hpf) were similar to those for the reference standard EoE cases (Table 1).

Table 3 Sensitivity and specificity of the case definitions[1] *n* (%)

	Development set		Validation set	
	Sensitive definition	Specific definition	Sensitive definition	Specific definition
Sensitivity	90 (90)	38 (38)	88 (88)	21 (21)
Specificity	60 (60)	97 (97)	80 (80)	96 (96)

[1]Sensitivity: Number of true positive eosinophilic esophagitis (EoE) cases identified by the case definition divided by the total number of reference standard EoE cases; Specificity: Number of true negative subjects identified by the case definition divided by the total number of subjects without EoE.

When these algorithms were applied to the validation set, the clinical features for the sensitive definition (n = 26; mean age 49 years; 81% male; 69% dysphagia; 60 eos/hpf) and specific definition (n = 6; mean age 49 years; 67% male; 50% dysphagia; 43 eos/hpf) were also similar to those for the reference standard for EoE cases (Table 1).

In the development set, the "sensitive" algorithm had a sensitivity and specificity of 90% and 60%, and the "specific" algorithm had values of 38% and 97%, respectively (Table 3). When these algorithms were applied to the validation set, the operating characteristics were essentially unchanged, with a sensitivity and specificity of 88% and 80%, for the "sensitive" algorithm, and 21% and 96% for the "specific" algorithms.

DISCUSSION

The epidemiology of EoE has been rapidly evolving over the past two decades, with a marked increase in incidence and prevalence. The study of EoE in large national or administrative databases, however, has been hampered by the lack of a validated case definition of EoE and the difficulty of developing such a definition due to the clinicopathologic nature of the disorder. This study takes advantage of the fact that the health registries in Denmark can link pathologic data to diagnostic and prescription coding data, thus providing all of the needed components to identify cases of EoE.

There are two main results for this study. The first is that the confirmed EoE cases identified in Denmark *via* the health registries had similar features to those reported both in Denmark as well as elsewhere in the world[1,7-17,32-35]. The second is that two registry-based case definitions, one highly sensitive and one highly specific, were successfully developed and validated for use in the Danish national health databases. This is the first study to do so, and this result makes future large-scale population-based studies feasible in that country. This is important because the majority of investigations studying the epidemiology of EoE are either from selected counties within a country or region, from single centers but are not population-based, or from larger databases that have limitations.

For example, in the two counties studied in northern Sweden during the Kalixanda study, Ronkainen *et al*[36] found that 11 of 1000 (1.1%) subjects had prevalent esophageal eosinophilia ⩾ 15 eos/hpf. While this is the only published study with a true population-based sampling strategy, these individuals would not necessarily meet current EoE diagnostic guidelines because not all were symptomatic and competing causes of esophageal eosinophilia were not excluded[1,28]. In Olten County, Switzerland, a well-defined geographic region with a stable population and practitioners who are expert in EoE diagnosis, Hruz *et al*[14] have recently updated their estimates of the incidence (7/100 000) and prevalence (43/100 000) of EoE[12]. Prasad *et al*[9] reported similar estimates from Olmstead County, Minnesota (incidence 9/100 000; prevalence 55/100 000) in patients identified retrospectively, and Spergel *et al*[37] derived a similar prevalence estimate (52/100 000) from physician surveys. In all of these studies, however, the included patients and providers were not sampled in population-based frames. Additional retrospective single center studies, while providing important data, are subject to similar limitations[7,8,10,11,13,38,39]. Three prospective studies examining the prevalence of EoE in patients undergoing upper endoscopy for any symptom[17], or for dysphagia provide equally important data[15,16], but are also from single centers and enrolled patients who were actively undergoing evaluation and so cannot necessarily be generalized to an entire population. In a series of studies that utilized a large pathology database in the United States, the national distribution of esophageal eosinophilia and EoE has been confirmed, but the data are also not population based[40-42]. Most recently, abstract data from the national health system in the Netherlands has been presented showing a rapid rise in the incidence of esophageal eosinophilia, but this study did not employ a validated case definition of EoE[43]. The current study attempts to address the challenges encountered in the definition of EoE when using county- or national-level database or registry data, and to set the stage for conducting true population-level EoE research.

There are both limitations and strengths of this study that should be acknowledged. The first issue is whether there could be misclassification of cases of EoE in the reference standard groups. This appears to be unlikely given that histology, symptom coding, and prescription data were used to apply EoE consensus diagnostic guidelines to the study population. Nevertheless, because this is a retrospective analysis, we cannot completely exclude the possibility of overlap between gastroesophageal reflux disease and EoE, and are unable to fully address the issue of PPI-responsive esophageal eosinophilia (PPI-REE). It is interesting to observe, however that only 41% and 49% of subjects with esophageal eosinophilia were confirmed to have EoE in the development and validation sets, respectively, suggesting that half or more of subjects with esophageal eosinophilia do not have EoE. The proportion of subjects with esophageal eosinophilia who did not have EoE in our study is similar to the reported proportion of subjects with esophageal eosinophilia who have PPI-REE, ranging in various studies from approximately 30%-75%[44-48]. The poor specificity

of the presence of esophageal eosinophilia for the diagnosis of EoE, coupled with the high proportion of subjects with esophageal eosinophilia who have PPI-REE, emphasize that esophageal eosinophilia alone is not adequate for case-finding of EoE in pathology databases, and cannot be used in isolation to diagnose EoE[1,28,49,50]. A related point is that while it is a strength of this study to have obtained and re-reviewed the original pathology slides, it is important to acknowledge that the biopsies were originally taken for clinical purposes at the discretion of individual endoscopists, and the location and number of biopsies could vary.

It is a strength of the study that two case definitions, one sensitive and one specific, were developed. In instances where overlap or misclassification may be a concern, the "specific" algorithm could be used to ensure the most homogenous EoE population is generated. The two case definitions also allow for bounding and performing sensitivity analyses around epidemiologic estimates. Moreover, the use of the linked Denmark databases is another strength of this study, as there are relatively few data sources that are as rich and contain patient-level pathology information linked to administrative coding. This is illustrated by our ability to obtain and re-review the original glass pathology slides in order to validate the SNOMED codes for esophageal eosinophilia.

The case definitions developed in this study are derived from the Denmark databases and it is unknown whether these would be valid in other settings. However, the methodology presented here could be readily replicated in different databases to validate EoE case definitions, and our proposed algorithm could be used as a starting point in an iterative process to develop case definitions in other databases.

In conclusion, this study of the linked Danish national health registries successfully identified and confirmed cases of EoE in Denmark. Two administrative registry-based case definitions, one highly sensitive to maximize the number of cases of EoE identified, and one highly specific to minimize the number of false positive EoE cases included, were developed and validated. The operating characteristics of the case algorithms are sufficient to support future population-based studies of the epidemiology of EoE in Denmark, and may serve as a template for developing similar definitions in other databases.

COMMENTS

Background

Population-based data on eosinophilic esophagitis (EoE) are lacking. The national medical registries in Denmark offer a unique opportunity to systematically study EoE because these resources link pathology, clinical, and pharmacological data at the patient level. However, no validated registry-based case definitions of EoE exist, and these must be developed prior to conducting large-scale studies.

Research frontiers

EoE is a newly emerging chronic immune-mediated disorder characterized by symptoms of esophageal dysfunction and infiltration of the esophageal mucosa by eosinophils. The epidemiology of EoE is increasingly understood, but much of the data are from single referral centers, and there are few population-based studies published.

Innovations and breakthroughs

This is the first study to systematically develop and validate an administrative case definition of EoE in the Danish databases. The authors present two registry-based definitions, one highly sensitive and one highly specific for EoE.

Applications

These definitions can now be utilized in future population-based studies of EoE in Denmark.

Peer review

This is a well designed study about development and validation of a registry based case definition of EoE in Northern Denmark. It has to be pointed out that in the re-reviewed slides of pathology represents an advantage in methodology.

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BRIEF ARTICLE

Endoscopic ultrasound evaluation in the surgical treatment of duodenal and peri-ampullary adenomas

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Abstract

AIM: To investigate endoscopic ultrasound (EUS) for predicting depth of mucosal invasion and to analyze outcomes following endoscopic and transduodenal resection.

METHODS: Records of 111 patients seen at our institution from November 1999 to July 2011 with the post-operative pathological diagnosis of benign ampullary and duodenal adenomas were reviewed. Records of patients who underwent preoperative EUS for diagnostic purposes were identified. The accuracy of EUS in predicting the absence of muscular invasion was assessed by comparing EUS reports to the final surgical pathological results. In addition, the incidence of the post-operative complications over a period of 30 d and the subsequent long-term outcome (recurrence) over a period of 30 mo associated with endoscopic and transduodenal surgical resection was recorded, compared and analyzed.

RESULTS: Among 111 patients with benign ampullary and duodenal adenomas, 47 underwent preoperative EUS for 29 peri-ampullary lesions and 18 duodenal lesions. In addition, computed tomography was performed in 18 patients, endoscopic retrograde cholangio-pancreatography in 10 patients and esophagogastroduodenoscopy in 22 patients. There were 43 patients with sporadic adenomas and 4 patients with familial adenomatous polyposis (FAP)/other polyposis syndromes. In 38 (81%, $P < 0.05$) patients, EUS reliably identified absence of submucosal and muscularis invasion. In 4 cases, EUS underestimated submucosal invasion that was proven by pathology. In the other 5 patients, EUS predicted muscularis invasion which could not be demonstrated in the resected specimen. EUS predicted tumor muscularis invasion with a specificity of 88% and negative predictive value of 90% ($P < 0.05$). Types of resection performed included endoscopic resection in 22 cases, partial duodenectomy in 9 cases, transduodenal ampullectomy with sphincteroplasty in 10 cases and pancreaticoduodenectomy in 6 cases. The main post-operative final pathological results included villous adenoma ($n = 5$), adenoma ($n = 8$), tubulovillous adenoma ($n = 10$), tubular adenoma ($n = 20$) and hyperplastic polyp ($n = 2$). Among the 47 patients who underwent resection, 8 (17%, 5 of which corresponded to surgical resection) developed post-procedural complications which included retroperitoneal hematoma,

intra-abdominal abscess, wound infection, delayed gastric emptying and prolonged ileus. After median follow-up of 20 mo there were 6 local recurrences (13%, median follow-up = 20 mo) 4 of which were in patients with FAP.

CONCLUSION: EUS accurately predicts the depth of mucosal invasion in suspected benign ampullary and duodenal adenomas. These patients can safely undergo endoscopic or local resection.

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Key words: Endoscopic ultrasound; Duodenal periampullary adenoma; Esophagogastroduodenoscopy; Cholangio-pancreatography

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INTRODUCTION

Duodenal tumors often pose diagnostic and therapeutic challenges in their management as they arise in close proximity to biliary and pancreatic structures[1]. Villous tumors of the duodenum was first described by Perry in 1893 as a broad-based cauliflower-like mass that he referred to as a duodenal papilloma and in a 1981 review, only 73 cases had been reported by Komorowski *et al*[2]. Although tumors that arise in the duodenum are not typically common (only 1% of gastrointestinal tumors) a significant fraction of them (at least 25%) are adenomas at the time of presentation. Treatment of ampullary adenoma is complicated by difficult preoperative staging, malignant potential and a high recurrence rate[3]. Various techniques are advocated for their management ranging from simple excision to the ampullary tumor and the contiguous duodenal mucosa to wide resection of the mass including the papilla and adjacent duodenal, ductal and pancreatic tissue[4]. Despite their benign nature peri-ampullary and duodenal adenomas remain a therapeutic challenge because of their potential for malignant transformation. Surgical resection of duodenal tumors can be particularly difficult because of their location in the retroperitoneal space in contact with normal pancreatic gland entailing a high risk of postoperative morbidity and mortality[5] . The complex anatomy of the ampullary region and the difficulty of accurately diagnosing and staging *via* imaging and endoscopy makes the management of these tumors controversial and require multidisciplinary teams[6]. While these tumors can typically arise anywhere along the duodenum, they predominately appear in the first or second portion with tumors in the 3rd and 4th portions being less frequent but posing more difficulty in endoscopic detection[1]. Accurate preoperative histological diagnosis and staging of these tumors is therefore often difficult and inconclusive leading to controversy over the ideal management of treating these lesions. Transduodenal local excision (TDE), endoscopic snare excision or pancreatoduodenectomy (PD) are valid options for resection of these tumors[6]. Halstead first reported TDE of an ampullary mass in 1899[7]. In 1935 Whipple was the first to perform *en bloc* removal of the entire duodenum with the head of the pancreas in 1935 a procedure later refined to a one-stage procedure called PD in 1940 by Whipple[8]. Some studies have reported that the incidence of malignancy occurring in duodenal or ampullary tumors vary widely with some reports ranging from 35% to 60% and report that while the region of the ampulla can be accessible for endoscopic biopsy procedure, that there is a high incidence of false negative results for carcinoma, ranging from 25% to 60%[7]. In 1990, a collective series of 78 Japanese patients reported that the biopsy diagnosis of adenoma does not rule out the possibility of deeper carcinoma in ampullary tumors[9]. Another case report in 1992 found EUS as an emerging useful adjuvant to the preoperative evaluation of patients for potential local resection due to its ability to accurately diagnose duodenal adenomas and therefore usefulness in treatment planning[10]. Based on these earlier reported high incidence of malignancy and unreliability of preoperative endoscopic diagnosis, optimal operative management of these tumors remains controversial[7]. We performed a retrospective review of patients with suspected benign duodenal tumors who underwent preoperative EUS to determine the accuracy of this technique in predicting the absence of muscular invasion and also to analyze outcomes associated with endoscopic and transduodenal surgical resection at our institution.

MATERIALS AND METHODS

Patients

Records of 111 patients evaluated and treated at the University of Alabama Birmingham from November 1999 to July 2011 with the post-operative pathological diagnosis of benign ampullary and duodenal adenomas were identified and reviewed retrospectively. There were 43 patients with sporadic adenomas and 4 patients with familial adenomatous polyposis (FAP)/other polyposis syndromes. Patients were 55% women and average age was 63 years old (Table 1.)

Imaging diagnostic modality used

Among 111 patients examined, 47 underwent preoperative EUS for diagnostic purposes (Figure 1). In addition, computed tomography (CT) was performed in 18 patients, endoscopic retrograde cholangiopancreatography in 10 patients and esophagogastroduodenoscopy in 22 patients.

The accuracy of EUS in predicting duodenal adenoma absence of muscular invasion was assessed by com-

Table 1 Summary of clinical data of patients with benign duodenal and peri-ampullary adenomas who underwent endoscopic ultrasound pre-operatively

Age, yr, mean ± SD (range)	63.4 ± 12.63 (25-90)
Sex	45% male; 55% female
Types of resection	Endoscopic (22); pancreaticoduodenectomy (6); transduodenal (10); partial duodenectomy (9)
Tumor location	Periampullary (29); duodenal (18)
Post operative final path	Villous adenoma (5); tubulovillous adenoma (10); tubular adenoma (20); hyperplastic polyp (2); chronic inflammation (1); adenoma (8); Reactive epithelial changes of normal small bowel mucosa (1)
EUS prediction	Absence of muscularis invasion (n = 5, P < 0.05); underestimated submucosal invasion (n = 4, P < 0.05); Accurately predicted depth of tumor invasion (n = 38, P < 0.05)

EUS: Endoscopic ultrasound.

Table 2 2 × 2 Contingency table

	Absence of muscularis involvement path	
	Yes[1]	No
Muscularis involvement EUS		
No	36	4
Yes[1]	5	2

[1]Positive predictive value: 28%; Negative predictive value: 90%; Sensitivity: 33%; Specificity: 87.8% (P < 0.05). EUS: Endoscopic ultrasound.

paring EUS reports and comparing it to final surgical pathological results. In addition, the incidence of post-operative complications over a period of 30 d and subsequent long-term outcome (recurrence) over a period of 30 mo associated with endoscopic and transduodenal surgical resection was recorded, compared and analyzed.

RESULTS

Location of tumors

Twenty-nine patients had peri-ampullary tumors defined by immediate proximity (within 2 cm) to the major duodenal papilla. The remaining 18 patients had adenomas elsewhere in the duodenum as described in Table 1.

Type of resection performed

Resection of the analyzed adenomas was performed endoscopically in 22 cases. Partial duodenectomy was performed in 9 cases, TDE with ampullectomy with sphincteroplasty in 10 cases and pancreaticoduodenectomy in 6 cases.

Final pathologic results

Postoperative final pathology results included villous adenoma (n = 5), adenoma (n = 8), tubulovillous adenoma (n = 10), tubular adenoma (n = 20) and hyperplastic polyp (n = 2), chronic inflammation (n = 1), reactive changes of normal small bowel mucosa (n = 1).

Long-term follow-up

After median follow-up of 20 mo, there were 6 local recurrences (13%) 4 of which developed in patients with FAP (Table 2). Among the 47 patients who underwent resection, 8 (17%, 5 of which corresponded to surgical resection) developed post-procedural complications, which included retroperitoneal hematoma, intra-abdominal abscess, wound infection, delayed gastric emptying and prolonged ileus. EUS reliably identified absence of submucosal and muscularis invasion in 38 (81%) (P < 0.05) patients. There were 4 patients in whom EUS under-estimated invasion of the deep layers which was subsequently demonstrated in the final pathological analysis. In 5 cases (3 of which underwent pancreaticoduodenectomy, 1 underwent endoscopic resection, and 1 underwent transduodenal resection) EUS over-estimated submucosal invasion as this feature could not be proven in the resected specimen. Overall analysis therefore demonstrated that EUS predicted tumor muscularis invasion with a specificity of 88% and negative predictive value (NPV) of 90% (P < 0.05).

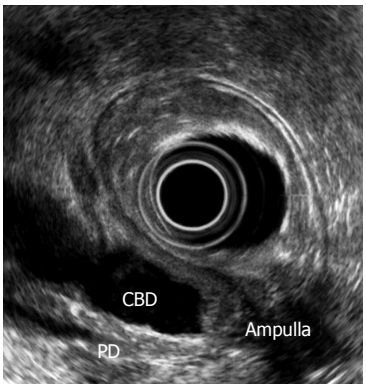

Figure 1 Endoscopic ultrasound showing absence of muscularis invasion in this lesion and as such amendable to local resection. PD: Pancreatoduodenectomy; CBD: Common bile duct.

DISCUSSION

The characterization of benign duodenal and peri-ampullary tumors, offers a diagnostic challenge to reliably distinguish adenomas from malignant lesions and render the possibility of transduodenal resection. While duodenal tumors are not common accounting for less than 1% of total gastrointestinal tumors and 25% of them are benign, the precise characterization continues to present a diagnostic challenge secondary to the complexity of the involved anatomical region. The surgical management of duodenal tumors is also challenging largely due

to their close relationship with the pancreatic and biliary ducts and their deep location[5].

The incidence of malignancy occurring in duodenal or ampullary tumors varies widely with false negative results for carcinoma as high as 60%[7] in the peri-ampullary region.

PD is the treatment of choice for invasive malignancies arising in the ampullary region and duodenum but its indication for treatment of benign peri-ampullary lesions is less clear. Several studies indicate that TDE exhibits less mortality and morbidity than PD[11]. Ampullectomy has been recommended as the procedure of choice to resect benign lesions smaller than 3 cm. TDE is therefore an organ-preserving operation with low morbidity but careful attention to the complex nature of the anatomy of the peri-ampullary region must be given to maximize its chances of success[11]. EUS is reportedly helpful in identifying non-invasive lesions suitable for local resection, but no preoperative test has been proven accurate enough to substitute for clinical judgment and intraoperative pathological confirmation[12]. In a study of local resection for ampullary tumors, the findings recommend local ampullary resection as an acceptable treatment in benign and selected premalignant and malignant ampullary lesions with a low threshold for conversion to PD when appropriate[13]. Another study of 63 patients proposed PD for even benign lesions because two patients in their series had to undergo repeat operations (PD) 4 and 22 years later for stage Ⅳ disease[14]. Local resection has been shown to be a viable alternative to PD in patients with benign tumors or as a palliative procedure in malignant cases with severe co morbidities where radical resection carries unacceptable surgical risk[15]. A review of 19 cases of villous tumors of the duodenum, suggests that some small benign ampullary villous adenomas or those with carcinoma in situ can be excised locally but prefer PD in the fit patient for better local control both of extensive benign lesions and cancers without distant metastases[16]. Other proponents of local resection confirm that benign duodenal villous tumors can be managed successfully by local submucosal excision[17]. In selected patients, endoscopic mucosal resection of superficial neoplastic lesions is associated with low morbidity when compared to surgery[18]. Pre-operative evaluation of these tumors to assess tumor depth is therefore paramount when planning the optimal therapeutic approach for resection of benign duodenal and peri-ampullary tumors. EUS has emerged as a useful technique in assessing the depth of invasion and is often employed when planning therapeutic approach. EUS images of tumors of the duodenal papilla correspond well to the final histological findings and report EUS as a reliable procedure for determining the extent of tumors in this location[19]. Comparison of EUS with other imaging modalities such as conventional sonography, CT, and angiography proved it to be the most effective method for local staging of pancreatic and ampullary cancers[20]. EUS can readily detect the presence of an ampullary or duodenal tumor in 96% of cases and reliably characterizes malignant lesions[21,22].

Consistent with prior publications, 16 of the 18 duodenal tumors in our study had tumors in the first or second portion of the duodenum (Table 1). Considering the above stated advantages, EUS is often employed at our institution when planning therapeutic approach to these tumors (Figure 1). Multiple studies have shown that EUS is superior to CT, magnetic resonance imaging, and transabdominal US in local peri-ampullary staging[23,24]. Previous studies evaluating the role of endoscopic resection of ampullary adenomas have presented it as a reasonable alternative to transduodenal surgical excision but long term follow-up data are needed to evaluate pre-operative staging accuracy and recurrence rates[25,26].

Our retrospective review shows that patients with peri-ampullary tumors and EUS showing absence of muscularis invasion can safely undergo transduodenal ampullectomy with sphincteroplasty and that this procedure results in satisfactory long-term outcomes. Muscularis invasion and pancreatic duct dilatation are features of malignant neoplasms that can be safely ruled out by EUS. Our findings further support endoscopic resection and TDE as safe treatment modalities for benign duodenal adenomas that avoid morbidity associated with PD and are associated with satisfactory long-term outcomes.

We conclude that EUS can accurately predict depth of mucosal invasion in the preoperative evaluation of suspected peri-ampullary and duodenal adenomas. These patients can safely undergo endoscopic or local resection with acceptable local control rates sparing the need for more extensive operations.

COMMENTS

Background

Management of benign duodenal and peri-ampullary adenomas is controversial as to the best management strategy. Some proponents of a more radical approach advocate a more aggressive pancreaticoduodenectomy while other proponents are in favor of a less invasive local resection.

Research frontiers

Endoscopic ultrasound (EUS) has emerged as a useful modality in the preoperative evaluation of these lesions and is used at our institution to guide management planning.

Innovations and breakthroughs

Duodenal tumors often pose diagnostic and therapeutic challenges in their management as they arise in close proximity to biliary and pancreatic structures and EUS has been shown to be a useful means of preoperative evaluation of these lesions to guide with the management planning. In one study, the sensitivity of EUS in the diagnosis of benign tumors was reported as 92%. The management of these benign tumors continues to be controversial posing a challenge to the surgeon, endoscopist and patients.

Applications

Based on their results, EUS can accurately predict depth of mucosal invasion in 81% ($P < 0.05$) of benign ampullary and duodenal adenomas with a specificity of 88% ($P < 0.05$) and negative predictive value of 90% ($P < 0.05$). These patients can safely undergo endoscopic or local resection with acceptable local control rates sparing the need for more extensive operations.

Terminology

Endoscopic ultrasound is an imaging modality that is used mostly in the upper digestive tract and in the respiratory system. It involves the insertion of a probe into a hollow organ and using ultrasound it is able to obtain images of internal organs that are in the chest, and abdomen and aids in visualizing these structures as well as blood vessels adjacent to them using the doppler imaging.

Gastroenterologists with advanced training typically perform the procedure. Transduodenal ampullectomy with sphincteroplasty is a local surgical excision procedure that is less invasive than the standard pancreaticoduodenectomy which is a radical resection typically used for more invasive lesions.

Peer review

This is a unique collection of cases establishing the value of endoscopic ultrasound in the management of duodenal and peri-ampullary adenomas and also reviewing the suitability and the results of the various possible operative procedures. With attention to minor details, it is an important paper and well worth publishing.

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BRIEF ARTICLE

Effect of alcohol consumption on liver stiffness measured by transient elastography

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Abstract

AIM: To determine the evolution of transient elastography (TE) in patients with alcoholic liver disease according to alcohol cessation or continuation.

METHODS: We retrospectively selected in our local database all patients who had two TE between June 2005 and November 2010 with chronic alcohol excessive consumption and excluded those with associated cause of liver disease. TE was performed at least one week apart by senior operator. TE examinations with less than ten successful measures or with an interquartile range above 30% were excluded. We retrospectively reviewed file of all patients to include only patient followed up by trained addictologist and for which definite information on alcohol consumption was available. Concomitant biological parameters [aspartate amino transferase (AST), alanine amino transferase and gamma-glutamyl transpeptidase (GGT)] within 4 wk of initial and final TE were recorded. Putative fibrosis score according to initial and final TE were determined with available cut-off for alcoholic liver disease and hepatitis C. Initial and final putative fibrosis score were compared according to alcohol consumption during follow-up.

RESULTS: During the study period 572 patients had TE examination for alcoholic liver disease and 79 of them had at least two examinations. Thirty-seven patients met our criteria with a median follow-up of 32.5 wk. At the end of the study, 13 (35%) were abstinent, and 24 (65%) relapsers. Eight patients had liver biopsy during follow-up. TE decreased significantly during follow-up in 85% of abstinent patients [median (range): -4.9 (-6.1,-1.9)], leading to a modification of the putative fibrosis stage in 28%-71% of patient according to different cut-off value. In relapsers TE increased in 45% and decreased in 54% of patient. There was no statistical difference between initial and final TE in relapsers. In the overall population, using 22.6 kPa as cut-off for cirrhosis, 4 patients had cirrhosis at initial TE and 3 patients had cirrhosis at final TE. Using 19.5 kPa as cut-off for cirrhosis, 7 patients had cirrhosis at initial TE and 5 patients had cirrhosis at final TE. Using 12.5 kPa as cut-off for cirrhosis, 16 patients had cirrhosis at initial TE and 15 patients had cirrhosis at final TE. Evolution of biological data was in accordance with the relapse or abstinent status: abstinence ratio (duration of abstinence/duration follow-up) was correlated with AST ratio (r = -0.465, P = 0.007) and GGT ratio (r = -0.662, P < 0.0001). GGT was correlated with initial (r = 0.488, P = 0.002) and final TE (r = 0.49, P < 0.005). Final TE was correlated with AST (r = 0.362, P < 0.05). Correlation between TE ratio and AST ratio (r = 0.44, P = 0.01) revealed that TE varied proportionally to AST for all patients irrespective of their alcohol status. The same relationship was observed between TE ratio and

GGT ratio (r = 0.65, P < 0.0001). Evolution of TE was significantly correlated with the ratio of time of abstinence to observation time (r = -0.387, P = 0.016) and the evolution of liver enzymes.

CONCLUSION: TE significantly decreased with abstinence. Results of TE in alcoholic liver disease cannot be interpreted without taking into account alcohol consumption and liver enzymes.

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Key words: Alcohol; Transient elastography; Cirrhosis; Fibrosis; Liver biopsy; Liver stiffness

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INTRODUCTION

Alcohol excessive consumption is a major public health issue[1,2] as it may lead to liver fibrosis and cirrhosis with life threatening complications such as hepatocellular carcinoma[3], liver failure and death[4]. Early diagnosis of cirrhosis is an important goal as it may promote change in alcohol consumption by increasing motivation in patients and lead for specific screening of esophageal varices[5] or hepatocellular carcinoma[6]. Diagnosis of cirrhosis can be easy when patients have clinical[7] or biological signs of portal hypertension or liver failure. But at the early stage, these signs are for the most part absent, and liver biopsy is the only way to assess fibrosis[8]. Cost and complications of liver biopsy[9-11], leading to significant morbidity or mortality, urge the search for non invasive tools using clinical, biological or morphological finding to establish a probability of fibrosis[12-15].

Transient elastography (TE) is a reproducible and non-invasive test to assess liver fibrosis in chronic liver disease[16]. Initial studies in hepatitis C[17-19] had shown an accurate identification of patients with mild fibrosis or cirrhosis and TE is now part of the European Association for the Study of Liver (EASL) clinical guidelines for hepatitis C management[20].

Studies have also suggested correlation between TE and fibrosis in other chronic liver disease such as hepatitis B[21,22], primary biliary cirrhosis[23], primary sclerosing cholangitis[24], and non alcoholic steatohepatitis[25,26], but it then appears that disease-specific cut-off values for significant fibrosis or cirrhosis should be used[27,28].

Concerning alcoholic liver disease, two studies have shown that TE is correlated with fibrosis[27,28], but diagnosis of severe fibrosis or cirrhosis need the use of higher cut-off values than in other diseases, moreover there are some discrepancies between proposed cut-off values. Explanations offered are different spatial distribution of fibrosis and, that acute liver damage increases liver stiffness by different ways such as hepatocyte swelling, cholestasis, inflammation, and hepatocyte necrosis[29]. Thus excessive alcohol intake, with or without acute alcoholic hepatitis, could increase TE and lead to a false positive diagnosis of fibrosis. Of note, no information regarding patient alcohol consumption at time of fibrosis evaluation was available in the studies which proposed those cut-off values. Our hypothesis is that the alcohol consumption greatly influences TE and thus explains the difficulty to validate TE in alcoholic liver disease, but in the other hand it could make TE a useful tool in the follow-up of patient as an indicator of alcohol consumption beyond the sole fibrosis evaluation.

The aim of this study was to assess the evolution of TE during follow up of patients with alcohol excessive intake history in regards to alcohol withdrawal or continuation. Secondary objective was to determine correlation between biological parameters and TE modification.

MATERIALS AND METHODS

Patients and data

This is a retrospective study conducted in the department of Hepatology and Addictology, University Hospital, Rennes, France. In this unit, a measure of TE is routinely performed in patients hospitalized for alcohol withdrawal or compensated alcoholic liver disease. TE is measured with the Fibroscan® (Echosens, Paris) by a senior operator.

Internal memory of our Fibroscan® was screened for patients who had two TE between June 2005 and November 2010 for alcoholic liver disease. The two different TE examinations had to be performed at an interval of more than one week, ten successful measures were required with an interquartile range ⩽ 30% of the median value. Then we reviewed the patients files to collect information on their history, duration of alcohol withdrawal, and alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltranspeptidase (GGT) at the time (or up to 4 wk before or after) of the two TE.

Patients were excluded if (1) they presented other causes of chronic liver disease (hepatitis B or C, extrahepatic cholestasis, hemochromatosis); (2) information about alcohol consumption between the two TE were missing, or (3) liver tests at the time of the two TE were missing.

Patients' alcohol consumption during follow-up was assessed by self-declaration and corroborated by biochemistry evolution. Only patients followed by trained addictologists with definite information regarding alcohol consumption were included. We defined abstinence ratio as the duration of abstinence/duration of observation. In this study, patients were considered as abstinent if they were totally sober at least 90% of the time between the two TE.

TE initial (TEi) corresponds to the first TE, TE final

Table 1 Initial and final biological parameters and transient elastography and of patients according to their alcohol consumption status

	Abstinent (n = 13)		Relapser (n = 24)	
	Initial	Final	Initial	Final
Normal AST	8%	75%	8%	15%
AST (IU/L)	130 (39-374)	42 (15-118)	140 (16-472)	119 (17-749)
ALT (IU/L)	109 (34-387)	44 (13-163)	88 (18-266)	76 (9-329)
GGT (IU/L)	1414 (121-3405)	301 (17-1040)	874 (48-5749)	536 (53-1858)
GGT (kPa)	15.5 (10.9-24.3)	11.7 (4.8-22.5)	11.7 (4-34.8)	13.5 (4.2-26.4)
Age, yr	48 ± 9		44 ± 10	
Sex (M/F)	11/2		4/20	

Data are expressed by median (quartile range) and mean ± SD. ALT: Alanine amino transferase; AST: Aspartate aminotransferase; GGT: Gamma glutamyltranspeptidase; M: Male; F: Female.

Table 2 Transient elastography and histological results of patient with liver biopsy

Patient	TEi (kPa)	EASL stage	Liver biopsy initial	TEf (kPa)	EASL stage	Liver biopsy final	Alcohol status
1	33.8	F4		26.4	F4	F4	Relapser
2	34.8	F4		75	F4	F4	Relapser
3	10.2	F3	F4	20.6	F4		Relapser
4	21.3	F4		14	F4	F3	Relapser
5	9.9	F3	F1	4.6	F ≤ 2		Relapser
6	5.3	F ≤ 2		16.9	F4	F3	Relapser
7	20.3	F4		14.3	F4	F4	Abstinent
8	11.7	F3		13.6	F4	F4	Abstinent

Data are shown in chronological order. EASL: European Association for the Study of Liver; TEi: Transient elastography initial; TEf: Transient elastography final.

(TEf) to the second TE and ΔTE to the difference between TEf and TEi. To determine variation of TE, we also defined TE ratio as (TEf-TEi)/TEi.

The liver fibrosis stages of the patients were calculated according to TE using cut-off values from different publications or guidelines: EASL recommendations for hepatitis C: 8 kPa for F ≥ 3 and 12.5 kPa for F = 4; Nahon[6]: 12.9 kPa for F ≥ 3 and 22.6 kPa for F = 4; and Nguyen[7]: 5.9 kPa for F ≥ 1, 7.8 kPa for F ≥ 2, 11 kPa for F ≥ 3 and 19.5 kPa for F = 4. Liver fibrosis stage referred to Metavir classification[9]: stage 0 corresponds to the absence of fibrosis, 1 to the presence of periportal fibrotic extension, 2 to periportal septa; severe fibrosis corresponds to stages 3 (porto-central septa) and 4 (cirrhosis). We defined Δstage as the difference between the fibrosis stages calculated at the first and the second TE, according to the different publications.

When liver biopsy had been performed, fibrosis was classified according to Metavir score[30].

We defined ASTi and GGTi as biological data related to the first TE, ASTf and GGTf to the second TE. The AST ratio [(ASTf-ASTi)/ASTi] and the GGT ratio [(GGTf-GGTi)/GGTi] were calculated to determine variation of biological markers.

Statistical analysis

Demographic data were expressed as median and inter quartile range. Qualitative variables were compared using the χ^2 test or Fischer exact test, when appropriate. Quantitative variables were compared using the Mann Whitney U test or Wilcoxon test for paired values. Correlation between variables was assessed using Spearman's rank correlation coefficient.

All tests were two sided with a significance of 5%. Tests were performed using JMP® software version 9.0 SAS, Cary, NC.

RESULTS

Characteristics of patients

During the period of the study, 6160 TE examinations were performed, of which 572 for patients with alcohol liver disease and 79 of them had 2 examinations. Forty-two patients were excluded because of missing data (lack of biological data or definite information on alcohol intake). Thus 37 patients corresponded to our criteria and were considered for analysis.

There were 7 women and 30 men with a median age of 44 years (39-53.5 years). Median follow up time was 32.5 wk (15-85 wk). At the end of the observation time, 13 (35%) were considered as abstinent, and 24 as relapsers.

Results of TE measures and biochemistry are shown in Table 1. Patients had marked increase in liver enzymes. TEi was significantly correlated with GGTi (r = 0.488, P = 0.002), and TEf with ASTf (r = 0.362, P < 0.05) and GGTf (r = 0.49, P < 0.05).

Repartition of patient according to putative liver fibrosis stage was different depending on TE cut-off used (EASL[2], Nahon[6] or Nguyen[7]). Results are shown in Figure 1. Overall, 16 patients had a TE indicating liver cirrhosis according to EASL, 7 according to Nguyen and 4 according Nahon cut-off values during initial evaluation.

Liver biopsy was performed in 8 patients. Results are shown in Table 2. According to EASL cut-off, 2 relapsers were misclassified. Patient 5 had stage 1 fibrosis, with TEi indicating severe fibrosis and normal TEf; and patient 6 had stage 3 fibrosis with TEf indicating severe fibrosis and normal TEi.

Evolution of TE according to alcohol consumption during observation period

During the observation period, 85% of abstinent patients had a decrease of TE, whereas among relapsers 45% had an increase and 54% a decrease of TE (P = 0.05, χ^2 test).

In the abstinent group, TE decreased significantly (Wilcoxon test for paired values, P = 0.0085) [median ΔTE = -4.9 kPa (-6.1, -1.9)] whereas TEf and TEi were not significantly different in relapsers [median ΔTE = -0.4 kPa (-4.8, +4)]. Δ TE were significantly different between abstinent and relapsers patients (P < 0.05).

There were variations in the putative staging of fibro-

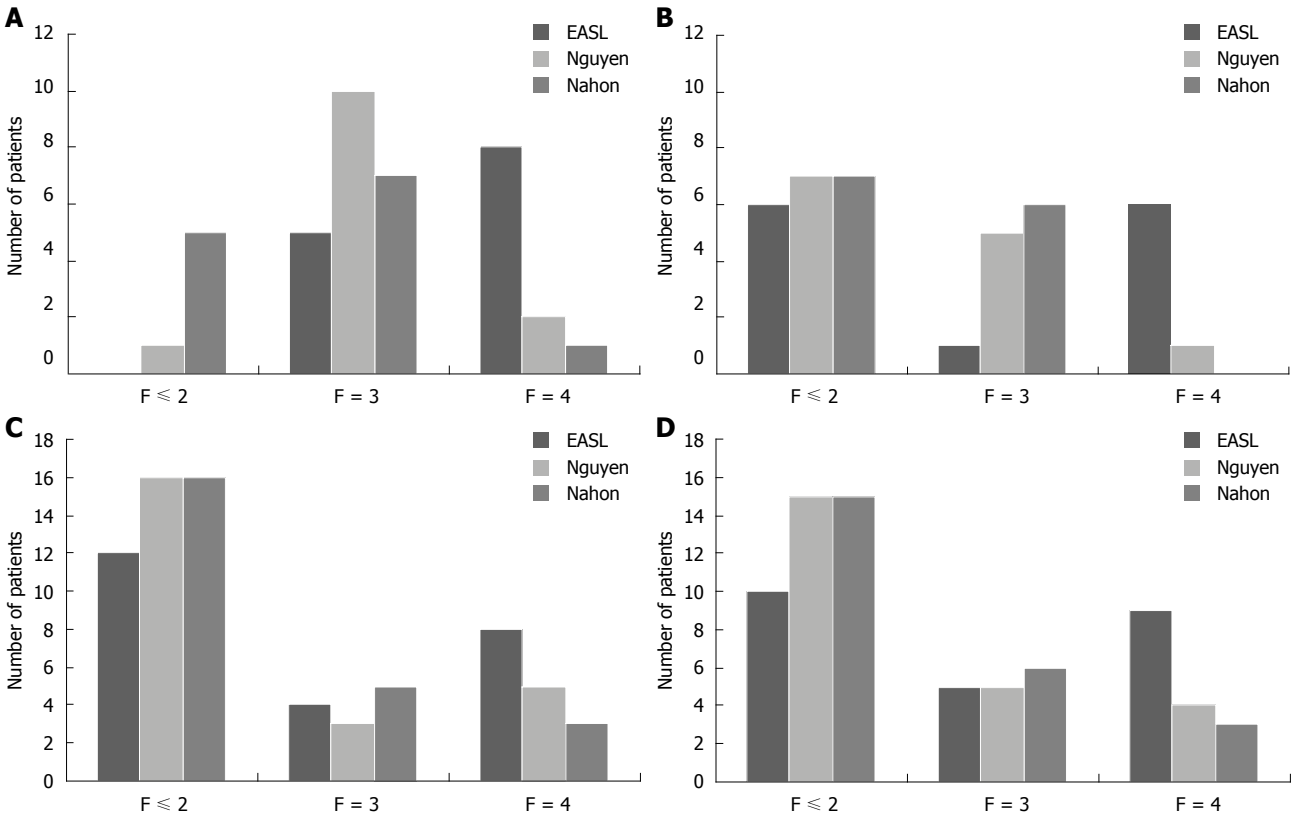

Figure 1 Initial and final repartition of liver fibrosis stage according to different cut-offs values of transient elastography (European Association for the Study of Liver, Nguyen and Nahon recommendations). A: Abstinent group [transient elastography initial (TEi)]; B: Abstinent group [transient elastography final (TEf)]; C: Relapser group (TEi); D: Relapser group (TEf). EASL: European Association for the Study of Liver.

sis according to TE during the observation time (Table 3). Putative fibrosis stage decreased in abstinent patients (in 28%-71% according to the cut-off used). In relapsers, stability, increase or decrease was observed. Δ stage according to EASL and Nguyen cut-offs were significantly different between relapsers and abstinent patients (P = 0.015 and P = 0.012 respectively).

TE decreased proportionally to abstinence, as demonstrated by the correlation between abstinence ratio and TE ratio (r = -0.47, P = 0.0029).

Variation of TE according to biological data

As expected abstinence ratio was correlated with AST ratio (r = -0.465, P = 0.007) and GGT ratio (r = -0.662, P < 0.0001).

Correlation between TE ratio and AST ratio (r = 0.44, P = 0.01) revealed that TE varied proportionally to AST for all patients irrespective of their alcohol status. The same relationship was observed between TE ratio and GGT ratio (r = 0.65, P < 0.0001).

DISCUSSION

TE is a useful tool which has been extensively validated in hepatitis C, but despite the incomparable more important frequency of alcoholic liver disease, few studies have been performed to assess TE in alcoholics. Moreover, despite of this active research, few studies evaluated TE regarding the current alcohol consumption of patients and only one studied the evolution of TE after alcohol detoxification. Our study is the first to assess TE evolution with a medium term follow up according to the alcohol consumption of patients.

Our results confirm the important variation of TE with alcohol withdrawal: 85% of abstinent patients showed a significant decrease of TE during a median follow up period of 32.5 wk. Moreover we showed a likely risk of overestimation of the fibrosis stage according to the different cut off values using TEi. TE evolution was correlated to AST and GGT evolution, which are usually used as marker of alcoholic hepatitis.

The results of our study may suffer from the retrospective data collection and the low number of patient. This is in part induced by the stringent inclusion criteria regarding missing data about alcohol consumption. Indeed, to avoid bias induced by poor quality of data and follow up bias we excluded all patients that were not followed by an addictologist and who lack definite information on alcohol consumption. Moreover, albeit alcoholic liver disease is an extremely frequent disease, patients are seldom compliant with follow up in the absence of severe disease, making rare those completing our inclusion criteria as shown by the number of TE performed for alcoholic liver disease (572) contrasting with the low number of patient who had a second TE (79).

In chronic hepatitis C, TE cut off associated with

Table 3 Percent of abstinent and relapser patient presenting a variation of Metavir score at the end of follow-up according to transient elastography using different cut off values

	Cut-off value	Increase in one or more stage of liver fibrosis (%)	Stable (%)	Decrease in one or more stage of liver fibrosis (%)
Abstinents	EASL	7	36	57
	Nguyen	7	22	71
	Nahon	7	65	28
Relapsers	EASL	29	55	16
	Nguyen	33	30	37
	Nahon	16	72	12

Results are expressed as percent of patient for each class. EASL: European Association for the Study of Liver.

fibrosis greater than stage 3 ranges from 8 to 9 kPa, and cirrhosis can be diagnosed with cut-off ranging from 14 to 15 kPa. Studies performed to assess TE diagnostic accuracy in other diseases than hepatitis C found higher cut off values for significant fibrosis or cirrhosis. Ganne-Carrié *et al*[31] studied 122 patients with non alcoholic or alcoholic steatohepatitis and found a cut off value for cirrhosis of 21.5 kPa. Nahon *et al*[27] studied 147 patients with alcoholic liver disease. Seventy-five percent of them had at least significant fibrosis, which is far more important than expected in alcoholic patients. They found a cut off value for significant fibrosis of 12.9 kPa and 22.6 kPa for cirrhosis. All the patients without cirrhosis and misclassified by TE had histological alcoholic hepatitis. In both studies, the patients were included if liver biopsy was indicated for chronic liver disease and there was no information regarding current alcohol consumption at the time of evaluation. Those data stressed the potential impact of alcohol induced liver modification beyond fibrosis on TE.

Coco *et al*[32] had reported that liver stiffness assessed by TE increased 1.3-to-3 fold during ALT flares in patients with viral hepatitis exacerbation. Studying 195 patients who had both liver biopsy and TE in acute liver damage, Fraquelli *et al*[33] showed that liver stiffness is increased in the acute phase with a correlation between TE and necroinflammatory activity. Recently Mueller *et al*[34] have showed that decrease of TE is correlated with the decrease of AST. Studying liver biopsies for alcoholic liver disease, they found that TE was constant if AST $\leqslant$ 100 UI/L and that accuracy of cirrhosis diagnosis by TE was improved in patient with AST $\leqslant$ 100 UI/L. Those data suggest that TE should be assessed differently regarding the current alcohol consumption and the presence or absence of acute liver modification or biochemical activity. In our study we used biochemical data gathered up to 4 wk from the date of TE, this is a long period which could have induced bias, however the rate of decrease or increase of TE in regard to the biochemical evolution remain to be determined in prospective study.

To date only one study assessed the evolution of TE with alcohol detoxification. Gelsi *et al*[35] studied in a population from an addictology unit the evolution of TE after alcohol weaning over a period of 60 d, and compared this evolution between relapser and abstinent patients. They found a rapid decrease of TE (-21% ± 27% at day 8) with detoxification in an increase proportion of patients if abstinence was sustained: 41% of patient had a decreased at day 8 and 66.7% at day 60. Relapsers were found to have a new increase in TE during follow up after alcohol relapse. As fibrosis is not likely to evolve during that short period of time, and albeit no liver biopsies were performed, one can assume that first TE measurement could have lead to overestimation of fibrosis.

Our results confirm those data, during a much longer follow up period (median 32.5 wk) with a precise addictologic follow up. TE decrease after alcohol cessation over a long period of time, and this was of particular importance in TEi ranging from 8 to 16 kPa, which could indicate significant fibrosis or cirrhosis in chronic hepatitis C, but should be interpreted with caution in alcoholic liver disease. However due to the retrospective design of the study which induced a variable timespan between TE examination, we could not assess the optimal time to perform TE after alcohol cessation.

Relapsers were found to have either an increase or a decrease of TE during follow up, this could be due to the level of alcohol consumption after relapse, as relapsers could have a lower alcohol consumption during follow up which could lead to a decrease of TE, as suggested by the correlation between Δ TE and GGT ratio. This point should be assessed in a prospective study with precise alcohol consumption amount, if confirmed TE could thus be a useful tool to monitor adherence during follow up and fluctuation in alcohol consumption.

TE was correlated with AST and GGT, and TE ratio was correlated with AST and GGT ratio, indicating that TE could be corrected by a calculated modifying factor based on liver enzymes value in order to increase the precision of this test in the diagnosis of fibrosis in alcoholic liver disease. This remains to be proven on large scale prospective studies of TE during alcohol withdrawal with liver biopsy as a gold standard. Such a study should also determine the optimal duration of alcohol cessation before initial increased TE could be controlled.

In conclusion our results show that TE decreased significantly after alcohol cessation over a long period of follow up. Thus TE in alcoholic liver disease should be interpreted with caution and assessed in regard to the current alcohol consumption. Variation of TE is correlated to AST and GGT suggesting that fibrosis could be more likely overestimated in patients with high biological perturbations. Large-scale prospective studies should be performed to determine the different optimal cut off values according to alcohol consumption and more data are required to determine the best delay of alcohol cessation for TE evaluation. TE examination could be a useful tool during the follow-up of alcoholic liver disease to assess actual alcohol consumption and maybe used as a prognosis tool as in chronic hepatitis C[36].

COMMENTS

Background

Excessive alcohol consumption is a major public health issue. Alcoholic liver disease can lead to liver fibrosis and cirrhosis with a risk of developing liver cancer or liver failure. Assessing the severity of liver fibrosis is thus an important landmark in alcoholic liver disease. Until recently the unique way to assess liver fibrosis was liver biopsy which is an invasive procedure with some risk of severe complication. Recently transient elastography (TE) was shown to be efficient in assessing liver fibrosis in chronic viral hepatitis C, it is now frequently used and acknowledged as a validated method by international hepatology society. Some studies have shown that TE could be used in alcoholic liver disease but with method differing from those in hepatitis C.

Research frontiers

TE (Fibroscan®) is an innovative non invasive method, measuring liver stiffness which is correlated to the severity of liver fibrosis. It has been widely validated in hepatitis C, but many studies have shown that results cannot be transposed "as this" to other type of liver disease because liver stiffness can be modified by other liver anomaly than fibrosis. In the area of alcoholic liver disease and liver fibrosis assessment, the research hotspot is how to use liver stiffness measured by TE and what are the parameter influencing liver stiffness.

Innovations and breakthroughs

This study showed that if performed in patient with alcoholic liver disease but without biological inflammation, performance of TE to assess fibrosis was good, which led the auhors to study the evolution of liver stiffness in patient with alcoholic liver disease in regard to their alcohol consumption.They compared the evolution of liver stiffness and biological data in patient with continued or stopped alcohol consumption. They showed that liver stiffness significantly decreases in patient after alcohol cessation and that this decrease is proportional to the severity of the biological inflammation.

Applications

The study results suggest that transient elastrography could be used in alcoholic liver disease by interpreting stiffness values in regard to the ongoing or not alcohol consumption. This hypothesis needs prospective confirmation before clinical application.

Terminology

Liver fibrosis is a "scar" that is developed in liver secondary to toxic (alcohol, iron overload) or viral (hepatitis C) damages. With increasing amount of fibrosis, cirrhosis appear thus leading to liver loss of function and an increased risk of liver cancer.

Peer review

The enclosed manuscript aims to the effect of alcohol consumption on liver stiffness measured by TE. The aim of the manuscript is sound.This is an interesting study.

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BRIEF ARTICLE

Role of Ki-67 as a prognostic factor in gastrointestinal stromal tumors

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Abstract

AIM: To investigate primarily the prognostic value of Ki-67, as well as other parameters, in gastrointestinal stromal tumors (GISTs).

METHODS: Ki-67, c-KIT, platelet-derived growth factor receptor-alpha (PDGFRα), smooth muscle actin (SMA), CD34, S100 were stained for immunohistochemistry which was performed on formalin-fixed, paraffin-embeded sections on representative block from each case. Proliferation index counted by Ki-67 antibody was calculated as a number of positive nuclear reaction over 100 cells. Immunoreactivity for c-KIT and PDGFRα was evaluated semiquantitatively (weak, intermediate, strong) and for c-KIT type of reactivity was analyzed (cytoplasmic, membrane and "dot-like" staining). Immunoreactivity for SMA, CD34 and S100 were was evaluated as positive or negative antigen expression. Pathologic parameters investigated in this study included tumor size, cell type (pure spindle, pured epitheloid mixed spindle and epitheloid), mitotic count, hemorrhage, necrosis, mucosal ulceration. Clinical data included age, gender, primary tumor location and spread of disease. χ^2 test and Student's *t*-test were used for comparisons of baseline characteristics. The Cox's proportional hazard model was used for univariable and multivariable analyses. Survival rates were calculated by Kaplan-Meier method and statistical significance was determined by the log-rank test.

RESULTS: According to the stage of disease, there were 36 patients with localized disease, 29 patients with initially localized disease but with its recurrence in the period of follow up, and finally, 35 patients had metastatic disease from the very beginning of disease. Tumor originated most commonly in the stomach (41%), small intestine was the second most common location (36%). The mean size of primary tumors was 6.5 cm. The mean duration of follow-up was 60 mo. Multiple parameters were analyzed for their effect on overall survival, but no one reached statistical significance (P = 0.06). Analysis of time to progression/relapse in initially localized disease (univariate analysis), tumor size, mitotic count, Ki-67 and type of d-KIT distribution (cytoplasmic *vs* membrane/"dot-like") showed statistically significant correlation. In multivariate analysis in the group of patients with localized disease, there were only 2 parameters that have impact on relapse, Ki-67 and SMA (P < 0.0001 and P < 0.034, respectively).

Furthermore, Ki-67 was analyzed in localized disease *vs* localized with recurrence and metastatic disease. It was shown that there is a strict difference between these 2 groups of patients (median value was 2.5 for localized disease *vs* 10.0 for recurrent/metastatic disease, $P < 0.0001$). It was also shown that the cut-off value which is still statistically significant in terms of relapse on the level of 6%. The curves for survival on that cut-off level are significantly different ($P < 0.04$, Cox F).

CONCLUSION: Ki-67 presents a significant prognostic factor for GIST recurrence which could be of great importance in evaluating malignant potential of disease.

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Key words: Gastrointestinal stromal tumors; Prognostic factor; Ki-67; Recurrence

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INTRODUCTION

Gastrointestinal stromal tumors (GIST) present a wide spectrum of tumors with variable malignant potential[1-3]. Although this entity has been defined rather recently, retrospectively it has been shown to be the most frequent mesenchymal neoplasm arising in the gastrointestinal tract[4]. Namely, in the last few decades, GISTs were sometimes wrongly diagnosed as leiomyoma, leiomyosarcoma and schwannoma. However, immunohistochemial staining and electron microscopic studies suggested difference comparing to other smooth muscle or Schwann cells. Finally, Hirota *et al*[5] demonstrated in 1998. mutation of c-KIT proto-oncogen as a paradigm of single-mutation tumorogenesis. It has also been shown that GIST originates from interstitial cells of Cajal[6].

Today we also know that both c-KIT and platelet-derived growth factor receptor-alpha (PDGFRα) oncoproteins are growth factor receptors that are normally activated by specific ligand stem cell factor. Their mutations lead to an independent activation of the receptor and constitutive activation and proliferation of cells[5]. Along with KIT-expression, very often is expressed CD34 and sometimes smooth muscle actin (SMA; focal of diffuse)[7].

The prediction of GIST behavior still remains controversial after all these years and, in many cases, non-conclusive. Many prognostic factors have been suggested and investigated in previous studies[8]. It is well known and established that the size of tumor (> 5 cm), mitotic rate > 5/50 HPF and site of the tumor play an important role. Nevertheless, it is not quite possible to predict the behavior of all GISTs. According to guidelines for the assessment of likely behavior, GISTs have been traditionally divided into probably benign, malignant and uncertain/low malignant potential (Fletcher)[9].

Imatinib mesylate, a selective tyrosine kinase inhibitor, has been known to have activity against such tumors. However, no patient had a complete response to the treatment so delineating prognostic factors for patients with GIST may be important even in post imatinib era[10]. It is not clear as well whether there is a role of adjuvant treatment in some GIST patients, although some current trials are still on going[11].

Ki-67 is a nuclear proliferation associated antigen. It is expressed in the growth and synthesis phases of the cell cycle but not in G0-phase (resting phase)[12]. It is a rather reliable marker of cell proliferation although there are differences between studies of Ki-67 labeling index, ranging from 4% to 22%[13,14].

The aim of this study was to investigate the role of Ki-67 among other parameters, as a prognostic factor of clinical behavior and prognosis, along with morphological and immunohistochemical profile in 100 GIST patients.

MATERIALS AND METHODS

All mesenchymal tumors of the gastrointestinal tract were retrieved from the files of the University Hospital Center Zagreb, in the period from 1997-2007. Since the immunohistochemistry has been developed on routine basis from the 2001, all specimens obtained before that time were retrospectively analyzed for c-KIT positivity. Inclusion criteria for the study were appropriate morphology and c-KIT positivity. Clinical data were retrieved from the files of the Clinic of Oncology and Surgical Oncology. Follow-up information was obtained from patients records or interviews. Clinical data included age, gender, tumor location (esophagus, stomach, small intestine, large bowel, retroperitoneum, mesentery) and spread of the disease. Spread of disease was classified into three groups: localized disease, localized disease with recurrence and metastatic disease. Pathological parameters that were assesed included tumor size, mitotic count, cell type (pure spindle, mixed spindle and epithelioid, pure epithelioid), necrosis, hemorrhagic areas and mucosal ulceration (invasion of lamina propria). Tumor size was measured in three dimensions and the largest dimension was taken into account. The mitoses were counted on 50 HPFx (400 ×). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections on representative block from each case. Following antibodies were used: CD-117 (Dako, Glostrup, Denmark), CD34 (DAKO), PDGFRα (Novocastra, England), SMA (SMA-1, DAKO), S-100 (DAKO), Ki-67 (MIB-5, DAKO). Positive controls were included. All samples were evaluated by two experienced pathologists. Immunoreactivity for c-KIT and PDGFRα was evaluated semiquantitatively (weak, intermediate and strong), and c-KIT type of reactivity was analyzed (cytoplasmic, membrane or "dot-like" staining). Proliferating index counted by Ki-67 antibody was calculated as a number of positive nuclear reaction over 100 cells. Immunoreactivity for SMA, S100 and CD34 was evaluated as positive or negative antigen expression.

Table 1 Patient distribution according to sex and age in three different groups

Groups		Sex		Total
		Male	Female	
Localized disease	Number	19	17	36
	Average age (yr)	59.5	62.9	61.1
Recurrent disease	Number	17	12	29
	Average age (yr)	55.5	58.4	56.7
Metastatic disease	Number	20	15	35
	Average	60.7	48.7	55.5
Total	Number	56	44	100
	Average age (yr)	58.7	56.8	57.9

Table 3 Multivariate analysis of different parameters for the disease free interval in patients with initially localized disease (n = 65)

Parameter	Beta	Standard error	t value	Exponent beta	P value
Tumor size	0.052	0.031	1.643	1.053	0.100
Number of mitosis	0.011	0.014	0.797	1.011	0.424
Necrosis	0.525	0.633	0.830	1.691	0.406
Haemorrhage	0.865	0.673	1.285	2.377	0.198
Ki-67	0.113	0.030	3.778	1.120	0.0001
c-KIT	0.592	0.536	1.102	1.807	0.270
SMA	-1.213	0.575	-2.110	0.297	0.034
S100	-0.237	0.771	-0.308	0.788	0.757
CD34	-1.088	0.562	-1.935	0.336	0.052

SMA: Smooth muscle actin.

Statistical analysis

χ^2 test or Student's t-test was used for statistical comparisons of baseline characteristics. In statistical analysis of our results we also used appropriate descriptive methods (median, range, minimum and maximum). The Cox's proportional hazard model was used in univariable and multivariable analyses. Survival rates were calculated by the Kaplan-Meier method, and statistical significance was determined by the log-rank test. The observed differences were assumed to be statistically significant if the probability of chance occurence was $P < 0.05$. All statistical analysis were performed by the statistical package statistica.

RESULTS

Our study comprised 100 GIST patients. Mean patients' age was 60.5 (range 20-78) years; 56% of patients were men. Patient distribution in three groups according to sex and age is shown in Table 1. There were 36 patients presenting initially with localized disease, 29 had localized disease further with recurrence and 35 had metastatic disease from the very beginning. Tumors originated most commonly in the stomach (41%), the small intestine was the second most common location (36%), in 8% colon and rectum and in 5% retroperitoneum was involved. In 10% of cases primary site of GIST was not clearly determined, because of the wide spread of the disease. The mean size of primary tumors (in patients without metastases) was 6.5 cm and 35 patients had distant metastases in the time of diagnosis. Metastases were most often localized in the liver, all other sites were rarely involved. The mean duration of follow-up was 60 (range 28-110) mo. Survival curve for all patients included in our study is shown in Figure 1. Further on, multiple parameters were analyzed for their effect on overall survival in all patients (Table 2). Most of them showed no effect, more precisely, only 2 parameters are close to statistically significant prediction of outcome and biological behavior, on the level of $P = 0.06$. These are Ki-67 and type of distribution of c-KIT. On the contrary, when we analyzed time to progression/relapse in localized disease, in univariate analysis tumor size, mitotic rate, Ki-67 and type of c-KIT distribution (cytoplasmic *vs* membrane/"dot-like") showed statistically significant correlation. In multivariate analysis in the group of patients with localized disease, there were only 2 parameters that have impact on relapse, Ki-67 and SMA expression (Table 3). Furthermore, when we compared Ki-67 in three different patients group it was obvious that there is a strict difference between mean value of Ki-67 in localized disease *vs* recurrent and metastatic disease together (median in localized disease was 2.5 *vs* 10.0 in recurrent and metastatic disease, $P < 0.0001$). It was shown that the cut-off value which is still statistically significant in terms of relapse on the level of 6% (Figure 2). Also, it has been shown that the curves for survival on that cut-off level are significantly different ($P = 0.04$, Cox F-test; Figure 3).

Table 2 Multivariate analysis of different parameters for survival in all analyzed patients (n = 100)

Parameter	Beta	Standard error	t value	P value
Tumor size	0.04	0.03	1.12	0.26
Mitosis	-0.01	0.01	-0.91	0.35
Necrosis	0.76	0.73	1.04	0.29
Haemorrhage	-0.63	0.68	-0.93	0.35
Ki-67	0.05	0.02	1.83	0.06
c-KIT	1.44	0.79	1.83	0.06
SMA	0.19	0.51	0.38	0.70
S100	-0.33	0.67	-0.49	0.62
CD34	-0.34	0.52	-0.66	0.50
Cell morphology	-0.19	0.52	-0.37	0.70

SMA: Smooth muscle actin.

DISCUSSION

According to data obtained from our study, like age, sex distribution and primary localization, they are comparable to previous data in literature[8,15,16]. GISTs are rather rare tumors with very interesting biological behavior and sometimes unpredictable clinical course[17-19]. Although many reports indicated that the size and mitotic count are the most important and reliable prognostic factors[7], it has become obvious that the primary tumor localization was also very important issue[17]. Some 10 years ago, DeMatteo *et al*[20] showed that only tumor size is reliable predictor of survival in multivariate analysis. Some other

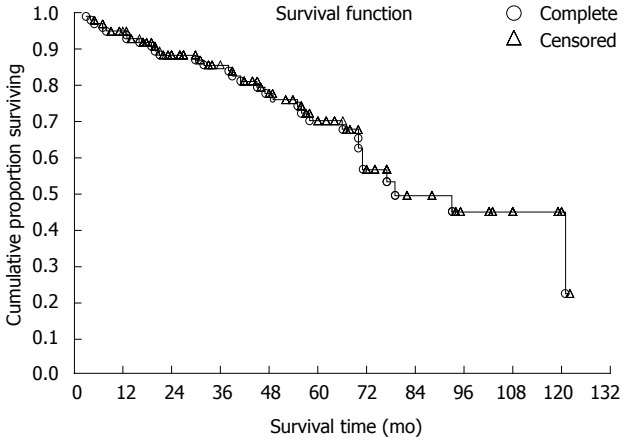

Figure 1 Survival curve for all patients with gastrointestinal stromal tumor (Cox regression analysis).

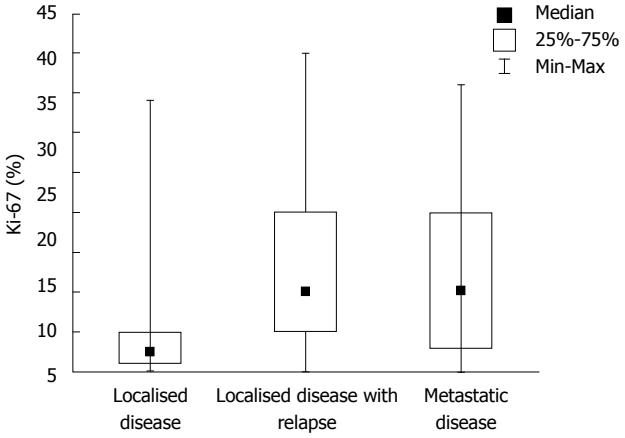

Figure 2 Value of Ki-67 in three different groups of patients.

authors found tumor size > 5 cm a poor prognostic factors[21]. However, there are only few reports of Ki-67 as a good indicator of the risk of metastases and as a prognostic factor[22]. Among other parameters, it was used for distinguishing leiomyoma and leiomyosarcoma[23]. Carrillo *et al*[13] stated that Ki-67 is one of the most accurate predictor of clinical behavior in GIST although it was not confirmed always and some other authors reported about Ki-67 with mitotic index as a prognostic factors in stomach GISTs[24]. In addition, we showed in our study, that there is no statistically significant difference in mean Ki-67 value between stomach- and small intestine-localization of GIST (52 patients together, 29 *vs* 23) (data not shown). That might support an idea of Ki-67 as a non-location-specific prognostic factor, what could be its advantage comparing to mitotic indeks which shows strong correlation to anatomic site of tumor.

One of the most important fact is observation that the level of Ki-67 is a prognostic factor for the relapse of initially localized disease ($P < 0.0001$), and the cut-off value is on 6%. Even in the survival setting this distinction remains statistically significant, no matter how these patients were treated ($P = 0.04$, Cox *F*-test). The expression of Ki-67 changes widely from one to another study, probably by the variety of cut-offs of various authors.

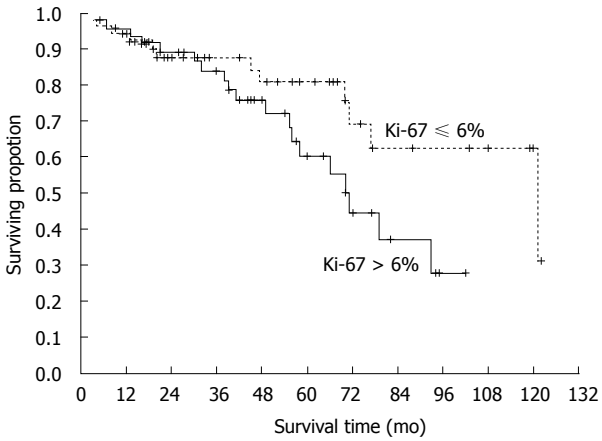

Figure 3 Survival curves with different values of Ki-67 (cut-off on 6%).

Toquet *et al*[25] and Nakamura *et al*[26] described cut-off of 10%, some others on the level of 5%. In that sense, our finding is important as additional indicator of importance of Ki-67 no matter the origin of GIST, and as well this might suggest, among other factors, which patient might have a greater risk of disease relaps. Maybe it might indicate the patients suitable for adjuvant treatment after surgical resection of localized GIST, in addition to contemporary concept of high-risk disease.

Considering other analyzed parameters, no one showed statistical power and impact on survival, as we expected. Just to mention that the meaning of statistical significance of SMA in DFI ($P < 0.034$) should be cautiously interpreted. It might be based on the fact that we assumed SMA-positive tumors generally better differentiated ones, thus it is to expect less aggressive course of disease which means fewer recurrences.

In conclusion, although there are some differences in numerical values of Ki-67 in various studies , that may be caused by different methodology in the assessment. But Ki-67 stays very important parameter of GIST-prognosis and should not be neglected when assessing malignant potential of the GIST.

COMMENTS

Background

Gastrointestinal stromal tumors (GIST) have a specific tumor biology with a wide spectrum of clinical behaviors. Thus, it is of great importance to define prognostic factors which could indicate risk of recurrence or spread of disease. Ki-67 is potentially good prognostic factor.

Research frontiers

So far, some of prognostic factors were rather well documented as the prognostic factors of further tumor behavior, for example mitotic count and tumor size, along with tumor primary localization. Ki-67 was unequivocally addressed as a prognostic factor.

Innovations and breakthroughs

This is the first study showing so clearly the significance of Ki-67 as a prognostic factor. Of importance is, that the patients included in the study were in different stage of disease, and also that comparison to other parameters have been done. It was shown that the cut-off value of Ki-67 of 6%, present the borderline value for recurrence of initially localized disease.

Applications

By understanding the meaning of Ki-67, it could be used as a parameter predicting tumor recurrence and suggest adjuvant treatment after surgery of localized disease.

Terminology

Ki-67 or MKI-67 is a protein that is in humans encoded by *MKI-67* gene. Ki-67 antigen is associated, and probably necessary, for cellular proliferation, it is associated with ribosomal RNA transcription. It is to assume that higher Ki-67 reveals tumor cell activity and thus predicts further behavior.

Peer review

This study is a contribution to the knowledge of GIST. It has a great value in better understanding of the biology of the disease, since some other parameters have already been recognized important as prognostic factors.

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BRIEF ARTICLE

Can endoscopic submucosal dissection be safely performed in a smaller specialized clinic?

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Abstract

AIM: To investigate whether endoscopic submucosal dissection (ESD) can be safely performed at small clinics, such as the Shirakawa Clinic.

METHODS: One thousand forty-seven ESDs to treat gastrointestinal tumors were performed at the Shirakawa Clinic from April 2006 to March 2011. The efficacy, technical feasibility and associated complications of the procedures were assessed. The ESD procedures were performed by five endoscopists. Sedation was induced with propofol for esophagogastorduodenal ESD.

RESULTS: One thousand forty-seven ESDs were performed to treat 64 patients with esophageal cancer (E), 850 patients with gastric tumors (G: 764 patients with cancer, 82 patients with adenomas and four others), four patients with duodenal cancer (D) and 129 patients with colorectal tumors (C: 94 patients with cancer, 21 patients with adenomas and 14 others). The *en bloc* resection rate was 94.3% (E: 96.9%, G: 95.8%, D: 100%, C: 79.8%). The median operation time was 46 min (range: 4-360 min) and the mean size of the resected specimens was 18 mm (range: 2-150 mm). No mortal complications were observed in association with the ESD procedures. Perforation occurred in 12 cases (1.1%, E: 1 case, G: 9 cases, D: 1 case, C: 1 case) and postoperative bleeding occurred in 53 cases (5.1%, G: 51 cases, D: 1 case, C: 1 case); however, no case required either emergency surgery or blood transfusion. All of the perforations and postperative bleedings were resolved by endoscopic clipping or hemostasis. The other problematic complication observed was pneumonia, which was treated with conservative therapy.

CONCLUSION: ESD can be safely performed in a clinic with established therapeutic methods and medical services to address potential complications.

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Key words: Endoscopic submucosal dissection; Complication; Perforation; Clinic

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INTRODUCTION

One-piece resection is considered the gold standard for endoscopic mucosal resection (EMR) because it provides an accurate histological assessment and reduces the risk of recurrence[1-5]. However, it is difficult to resect large and ulcerative lesions *en bloc* using conventional EMR techniques. Therefore, a new technique, endoscopic submu-

cosal dissection (ESD), has been developed[6-8]. ESD is a minimally invasive treatment for gastrointestinal cancer. It is usually performed in general hospitals because of the high frequency of complications and the need for a high level of technical skill[9-12]. The occurrence of complications negatively affects the quality life of patients and sometimes requires surgical treatment. Bleeding is one of the most common complications of ESD[13]. The use of hemostatic devices or endoscopic clipping prevents the occurrence of bleeding after ESD procedures[13]. Therefore, cases that require surgical treatment for bleeding after therapeutic procedures are quite rare. Perforation is also one of the most common complications of ESD[13]. Surgical treatment for iatrogenic perforation should be avoided at local clinics because most of these clinics do not have appropriate equipment for such surgeries.

The Shirakawa Clinic, a 19 bed inpatient clinic, was founded as a special clinic for endoscopic surgery. The main procedures performed at this clinic include endoscopy and endoscopic treatments. This clinic does not have surgeons or appropriate equipment for surgery. When surgical treatment is required, patients are transferred to a related tertiary hospital.

This study investigated whether ESD can be safely performed at small clinics such as the Shirakawa Clinic. The efficacy, technical feasibility and associated complications of ESD were assessed in the Shirakawa Clinic.

MATERIALS AND METHODS

Patients

We reviewed all cases of endoscopy and ESD to treat gastrointestinal tumors performed at the Shirakawa Clinic from April 2006 to March 2011. ESD was performed not only for treatment purposes, but also to make accurate histological diagnoses. Indications for ESD primarily included mucosal types of gastrointestinal cancers, excepting poorly differentiated cancers. A detailed description of the indications has been previously reported[9-12]. Depending on the procedure, we followed the guidelines of either the Japan Esophageal Society[14], the Japanese Gastric Cancer Association[15] or the Japanese Society for Cancer of the Colon and Rectum[16].

Clinic and ESD procedure

The Shirakawa Clinic includes 19 inpatient beds, 5 endoscopists and 5 endoscopical technicians certified by the Japan Gastroenterological Endoscopy Society. The endoscopic treatment room and treatment procedures are shown in Figure 1. The treatment teams included two doctors (one operator and one doctor who administered propofol sedation), two nurses and two endoscopical technicians. The years of experience of the endoscopists who performed the endoscopic treatment, including EMR, were as follows: 24 (Onozato Y), 20 (Sohara N), 16 (Hagiwara S), 14 (Iizuka H), and 11 (Arai R) years. The number of years of experience with ESD treatment were 10 (Onozato Y), 5 (Sohara N), 4 (Hagiwara S), 8 (Iizuka H), and 2 (Arai R) years. The numbers of ESD treatments performed were about 1000 (Onozato Y), 250 (Sohara N), 150 (Hagiwara S), 300 (Iizuka H), and 50 (Arai R) cases, respectively. All ESD procedures were supervised by Onozato Y, who had most experience performing ESD among the five endoscopists. As a result, all ESD cases were similarly treated because of the careful supervision.

The intravenous administration of pethidine hydrochloride and propofol was used for sedation, except in colorectal ESDs. The tumors were treated using standard ESD procedures as previously described[9-12]. For each patient, a data collection sheet was used to obtain relevant clinical information about the patient, tumor, procedure and complications. The data sheets were reviewed retrospectively. Findings from abdominal X-P, physical examinations and blood tests were assessed using a clinical pathway as a basis. Patients without complications were permitted to eat soft foods two days after ESD and then were discharged after endoscopic follow-up on the seventh day. The efficacy and complications of ESD were assessed.

One-piece resection was defined as *en bloc* resection. The resections were considered to be curative when tumor-free vertical and horizontal margins were achieved, *en bloc* resection was performed and resection criteria were met[14-16]. The operation time was calculated from the data of digital versatile disc recording systems for endoscopic procedures. Time from the start of the mucosal incision to the completion of the dissection was defined as the operation time. The time for marking or hemostasis after dissection was excluded from the operation time.

Procedure-related bleeding was subdivided into intra- and postoperative groups. Intraoperative bleeding was defined as large amounts of bleeding during the procedure and difficult cases of hemostasis. Postoperative bleeding was defined as clinical evidence of bleeding as evidenced by hematemesis or melena at 0-30 d after ESD and requiring endoscopic treatment. Furthermore, postoperative bleeding was subdivided into early postoperative (within three days of ESD) and delayed postoperative (more than three days after ESD) groups. We also recorded the incidence of perforation during endoscopy and after the procedures based on clinical evidence.

Endoscopic follow-up examinations were performed routinely one week, three months, nine months and every six months after ESD. To exclude distant metastases, abdominal ultrasonography (and/or computed tomography) was performed before ESD and three months and every six months after ESD on the same day as endoscopy.

Statistical analysis

The continuous variables are presented as the mean ± SD. The data were analyzed using the Fisher's exact probability test, and Mann-Whitney's *U*-test. A P value < 0.05 was considered to be significant.

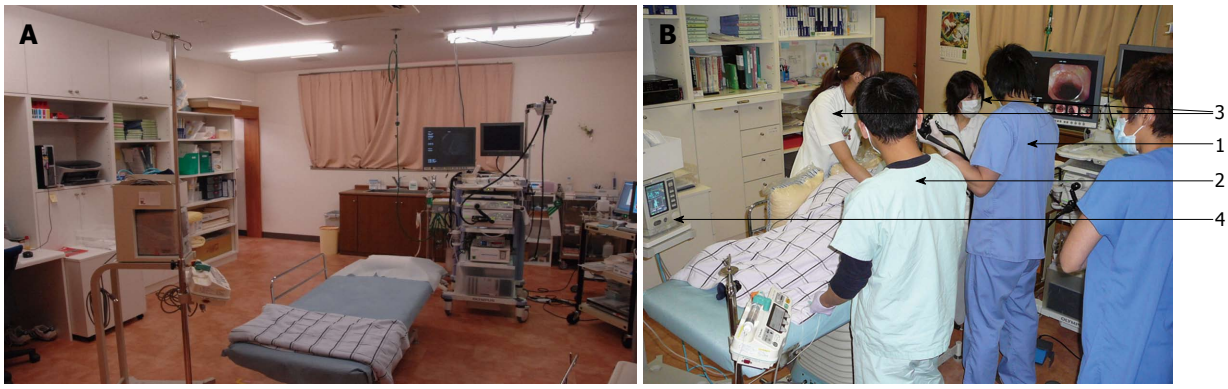

Figure 1 Endoscopic treatment room (A) and treatment procedures (B) of the Shirakawa Clinic. The treatment team included two doctors [one operator (1) and one doctor to administer propofol sedation (2)], two nurses who care for the patients (3) and two endoscopical technicians (not in photo). 4: Vital sign monitor.

Table 1 Clinical characteristics and major complications of the endoscopic submucosal dissection patients

	Total	Esophagus	Stomach	Duodenum	Colorectum
Patients, *n*	873	59	681	4	129
Age (yr), mean (range)	69.9 (44-91)	68.0 (46-81)	70.9 (45-91)	65.5 (54-77)	65.9 (44-80)
Sex (male:female), *n*	549:324	52:7	408:273	2:2	87:42
Lesions, *n*	1047	64	850	4	129
Tumor size (mm), mean (range)	22.2 (2-150)	23 (4-46)	20.8 (2-150)	16.8 (9-31)	31.6 (2-92)
Tumor diagnosis and depth, *n*					
Benign tumor	121	0	86	0	35
Cancer (m)	808	56	668	4	80
Cancer (sm1)	61	4	48	0	9
Cancer (sm2 or deeper)	57	4	48	0	5
En bloc resection rate, %	94.3	96.9	95.8	100	79.8
Operation time (min), mean (range)	46 (4-360)	62 (48-206)	42 (4-360)	90 (50-114)	60 (7-300)
Complications associated with ESD, *n* (%)					
Perforation	12 (1.1)	1 (1.7)	9 (1.1)	1 (25.0)	1 (0.8)
Postoperative bleeding	53 (5.1)	0 (0.0)	51 (6.0)	1 (25.0)	1 (0.8)

ESD: Endoscopic submucosal dissection.

RESULTS

Total patients

From April 2006 to March 2011, a total of 35 540 endoscopies were performed at the Shirakawa Clinic. A total of 1047 lesions in 873 patients were resected with ESD (Table 1). Sixty-four patients with esophageal cancer (E), 850 patients with gastric tumors (G: 764 patients with cancer, 82 patients with adenomas and four others), four patients with duodenal cancer (D) and 129 patients with colorectal tumors (C: 94 patients with cancer, 21 patients with adenomas and 14 others) were treated. The *en bloc* resection rate was 94.3% (E: 96.9%, G: 95.8%, D: 100%, C: 79.8%). The median operation time was 46 min (range: 4-360 min) and the mean size of the resected specimens was 18 mm (range: 2-150 mm). No mortal complications were observed in association with the ESD procedures. Perforation occurred in 12 cases (1.1%, E: 1 case, G: 9 cases, D: 1 case, C: 1 case) and postoperative bleeding occurred in 53 cases (5.1%, G: 51 cases, D: 1 case, C: 1 case); however, no cases required emergency surgery or blood transfusion. All of the perforations were resolved using endoscopic clipping or hemostasis. The other problematic complication observed was pneumonia, which was treated with conservative therapy.

Esophageal cancers

The follow-up results of the 64 patients with esophageal cancer are shown in Figure 2A. The median follow-up period was 24 mo (range: 12-62 mo). Forty-six patients met the absolute indication criteria of the guidelines issued by the Japan Esophageal Society in 2007[14]. Twelve patients with lesions did not meet the conventional absolute criteria and instead met expanded indication criteria. Four patients did not meet the indication criteria. The *en bloc* resection rate was 96.9%. One of the 12 patients who met the expanded indication criteria and two of the four patients who did not meet the indication criteria received additional treatment. No local recurrences were observed in the patients who met the indication criteria.

Gastric cancers and adenomas

The follow-up results of the 850 patients with gastric tumors (764 patients with cancer, 82 patients with adenomas and 4 others) are shown in Figure 2B. The median follow-up period was 26 mo (range: 12-62 mo). Four

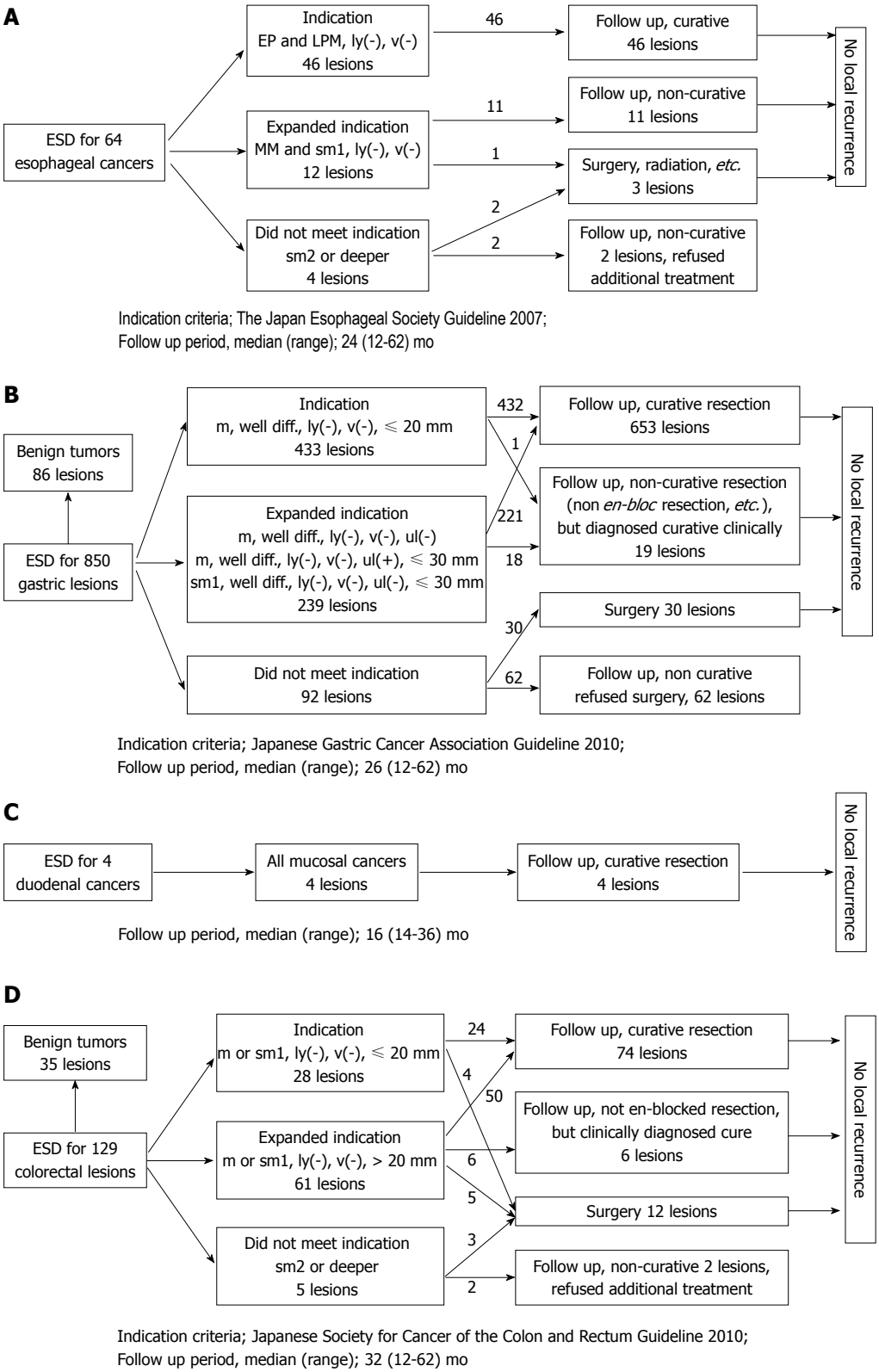

Figure 2 The clinical courses after the endoscopic submucosal dissection of esophageal cancers (A), gastric lesions (B), duodenal cancers (C) and colorectal lesions (D). ESD: Endoscopic submucosal dissection; EP: Epithelium; MM: Muscularis mucosae; LPM: Lamina propria mucosae; Well diff.: Well differentiated adenocarcinoma.

hundred and thirty-three of the 764 patients with gastric cancer met the absolute indication criteria of the guidelines issued by the Japanese Gastric Cancer Association in 2010[15]. Two hundred and thirty-nine patients with lesions did not meet the conventional absolute indication criteria and instead met expanded indication criteria. Ninety-two patients with lesions exceeded the indication criteria. The *en bloc* resection rate was 95.8%. Curative

Table 2 Operation times and tumor sizes in the cases with and those without complications (mean ± SD)

	Number of lesions	Size of treated lesions (mm)	Operation time (min)
Esophagus			
All lesions	64	23.0 ± 11.0	76.4 ± 45.4
No complication	58	23.0 ± 11.1	76.4 ± 45.7
Complication			
Perforation	1	24	57
Postoperative bleeding	0	ND	ND
Stomach			
All lesions	850	20.2 ± 13.4	63.2 ± 47.2
No complication	791	19.9 ± 13.2	62.1 ± 46.9
Complication			
Perforation	8	31.7 ± 14.7[a]	108.9 ± 45.7[a]
Postoperative bleeding	51	23.5 ± 15.5	73.1 ± 47.9[a]
Duodenum			
All lesions	4	16.8 ± 10.1	94.5 ± 29.9
No complication	2	20.0 ± 15.6	112.0 ± 2.8
Complication			
Perforation	1	10	104
Postoperative bleeding	1	17	50
Colorectum			
All lesions	129	31.6 ± 16.5	86.6 ± 66.4
No complication	127	31.3 ± 16.4	84.5 ± 64.5
Complication			
Perforation	1	35	135
Postoperative bleeding	1	65	300

[a]$P < 0.05$ *vs* no complication group. ND: No data.

resection was achieved in 653 of the 764 patients with gastric cancer (85.5%). Nineteen patients with lesions underwent non-curative resection (non *en bloc* resection was performed, *etc*.) but were diagnosed curative clinically. No local recurrences were observed in the patients who met the indication criteria or in those who underwent clinically curative resection.

Duodenal cancers

The follow-up results of the four patients with duodenal cancer are shown in Figure 2C. The median follow-up period was 16 mo (range: 14-36 mo). All four patients underwent curative resection, and the *en bloc* resection rate was 100%.

Colorectal cancers and adenomas

The follow-up results of the 129 patients with colorectal tumors (C: 94 patients with cancer, 21 patients with adenomas and 14 others) are shown in Figure 2D. The median follow-up period was 32 mo (range: 12-62 mo). Twenty-eight of the 94 patients with colorectal cancer met the absolute indication criteria of the guidelines issued by the Japanese Society for Cancer of the Colon and Rectum in 2010[16]. Sixty-one patients with lesions did not meet the conventional absolute indication criteria and instead met expanded indication criteria. Five patients with lesions exceeded the indication criteria. The *en bloc* resection rate was 79.8%. Curative resection was achieved in 74 of the 94 patients with colorectal cancer (78.7%). Six patients with lesions underwent non-curative resection (because non *en bloc* resection was performed, *etc*.) but were diagnosed curative clinically. No local recurrences were observed in the patients who met the indication criteria or in those who underwent clinically curative resection.

Complications and endoscopic treatment

No mortal complications were observed in association with the ESD procedures. Perforations occurred in 12 cases (1.1%, E: 1 case, G: 9 cases, D: 1 case, C: 1 case) and postoperative bleeding occurred in 53 cases (5.1%, G: 51 cases, D: 1 case, C: 1 case) (Table 1). No problematic complications related to sedation were observed. One patient who experienced postoperative pneumonia was treated with an infusion of antibiotics and recovered in one week.

Operation times and tumor size for the cases with and without complications are shown in Table 2. Because the number of esophageal, duodenal and colorectal cases complicated by perforation or bleeding was small, it was not possible to make a statistical comparison between a no complication group and a complication group. It was possible to analyze the cases of gastric lesions statistically. Tumor size and operation times in the gastric cases with perforations were significantly larger than those observed in the cases without complications ($P < 0.05$).

All perforations were managed endoscopically using endoclips, a patch of omentum or conservative therapy. None of the patients with perforations required further surgery, and each of these patients were hospitalized for an additional zero to two days. A representative case of perforation is shown in Figure 3. Briefly, after detecting a perforation hole, the air was removed using an endoscopic fiber. Next, endoscopic clipping was performed. When the omentum became visible through the perforation hole, we attempted to aspirate the omentum through the perforation hole and clip the omentum to the hole.

All cases of intraoperative bleeding were controlled with endoscopic procedures such as soft coagulation with hemostatic forceps (Coagrasper; FD-410LR or FD-410-QR, Olympus, Tokyo). No case of intraoperative bleeding required surgical intervention or blood transfusion. Postoperative bleeding occurred in 53 cases. Early postoperative bleeding (within three days of ESD) occurred in 25/53 cases and delayed postoperative bleeding (more than three days after ESD) occurred in 28/53 lesions. All cases of postoperative bleeding were controlled with endoscopic treatment (clipping and/or electrocoagulation). No case of postoperative bleeding required surgical intervention or blood transfusion. A representative case of bleeding treated endoscopically is shown in Figure 3. Briefly, bleeding from a post-ESD ulcer was detected. After removing the coagulation, the bleeding point was detected. Clips were used to achieve hemostasis endoscopically.

DISCUSSION

ESD allows for the treatment of large and ulcerative

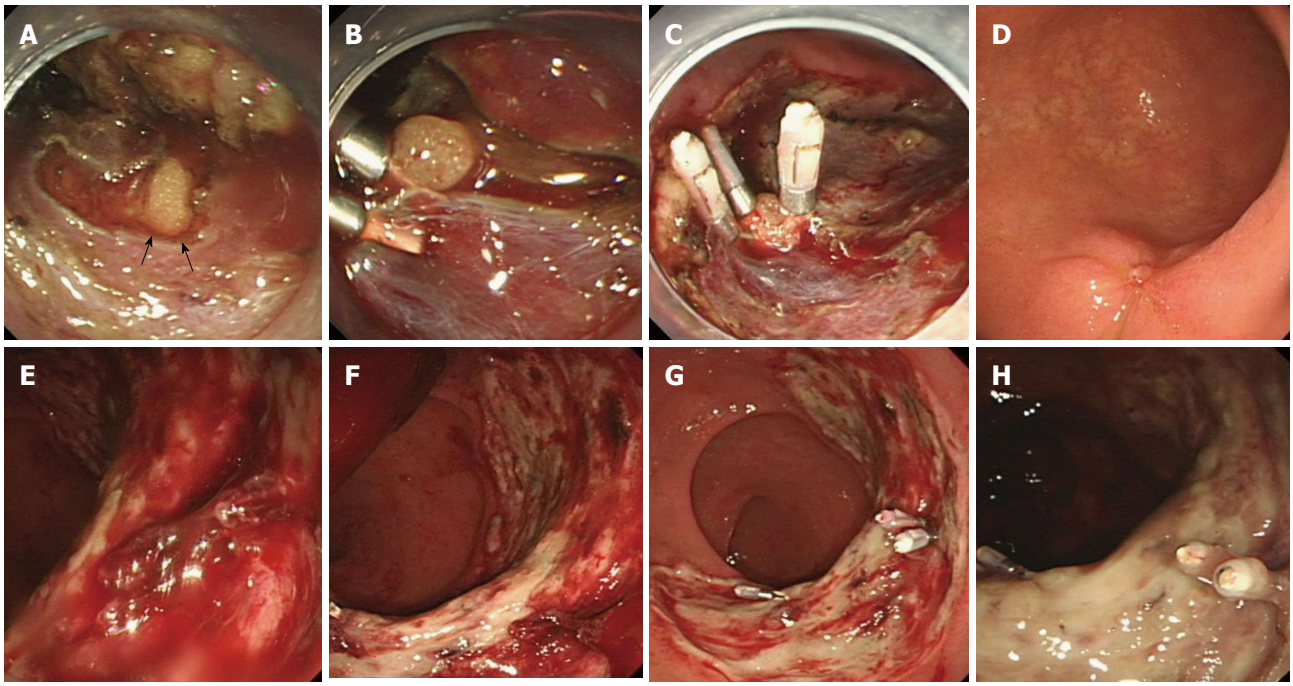

Figure 3 Representative cases of perforation and bleeding treated endoscopically. A: A perforation was detected (arrows); B: When the omentum became visible through the perforation hole, we attempted to aspirate the omentum through the perforation hole, then clipped the gastric wall and omentum together; C: Several clips were used to seal the hole completely; D: The appearance three months after clipping; E: Bleeding from a post-endoscopic submucosal dissection ulcer was detected; F: After removing the coagulation, the bleeding point was detected; G: Clips were used to achieve hemostasis endoscopically; H: The appearance 1 wk after clipping.

lesions and has revolutionized the management of gastrointestinal tumors[6,7,17,18]. ESD is a minimally invasive treatment for gastrointestinal cancer. However, ESD requires a high level of expertise and experience[19,20]. It is usually performed in general hospitals because of the high frequency of complications and the need for a high level of technical skill. Hemostatic devices and/or endoscopic clipping prevents the occurrence of complications after ESD. In this study, all complications of ESD were resolved using non-surgical therapies. Therefore, ESD can be safely performed in clinics with established therapeutic methods and medical services to address potential complications. This study showed that ESD can be safely performed in small clinics.

Since ESD is an endoscopic surgical procedure, the risk of complications is unavoidable[13]. Bleeding and perforation are most common complications of ESD procedures[13]. The incidence of complications[13] associated with ESD are reported to be: 0% for bleeding in the esophagus, 4.0%-6.0% for perforation of the esophagus[21,22], 1.8%-15.6% for bleeding in the stomach, 1.2%-4.7% for perforation of the stomach[23-26], 0.3%-1.5% for bleeding in the colon and 2.2%-5.5% for perforation of the colon[27-29]. Except in cases of esophageal ESD, bleeding is a common complication. It can be treated endoscopically using soft coagulation with hemostatic forceps or hemoclips[13]. In most reports, the incidence of bleeding is less than 10%[13]. In this study, the incidence of complications was 0% for bleeding in the esophagus, 1.7% for perforation of the esophagus, 6.0% for bleeding in the stomach, 1.1% for perforation of the stomach, 0.8% for bleeding in the colon and 0.8% for perforation of the colon. These complication rates are acceptable in comparison to those seen at other institutes[13].

Perforation is another common complication of ESD. Since mediastinitis and peritonitis occuring consequent to esophageal perforation and colon perforation, respectively, have high mortality rates, surgery was previously the method of choice for treating gastrointestinal perforations. Currently, conservative or endoscopic treatment tends to be selected to treat smaller perforations when the lesions are clean[30,31]. Most perforations resulting from ESD are smaller and tend to be treated in a non-surgical manner[32,33]. It is very important to recognize the occurrence of a perforation immediately. Endoscopic closure should be considered if the lesion is clean (i.e. without feces and so on). Matsui *et al*[13] have suggested the indications for endoscopic clipping of perforations to include (1) a small defect size (less than 10 mm); (2) the ability to prepare the bowel adequately; and (3) a stable patient condition immediate following perforation. After successful endoscopic closure is completed, the patient should be fasted and treated with antibiotics. Formal guidelines for fasting periods have not been established; however, one report regarding colonic perforations indicates a mean fasting period of 4.2 d and a mean duration of intravenous antibiotics of 5.5 d[34]. Surgical treatment should be considered when the perforated lesion cannot be closed with endoclips, abdominal pain has exacerbated or severe peritonitis is suspected. Of course, if there is any concern regarding the patient's condition, the patient should be transferred to surgery without hesitation. In

this study, all perforations were managed endoscopically using endoclips, patches of the omentum or conservative therapy. No patient required additional surgery due to perforation in this study.

Because ESD requires a high level of technical skill, an endoscopist's skill level[9-12], rather than the size of a clinic or hospital, may be more strongly related to the results of ESD, such as the success of *en bloc* resections, the length of operations and the rate of complications. In this study, all five endoscopists had more than 10 years of experience with endoscopic treatment, including EMR. Furthermore, all ESD procedures were supervised by the most experienced endoscopist in our clinic. As a result, all ESD cases received similar levels of treatment. This is one of the important reasons why ESD can be safely performed in small clinics. The patient volume may also strongly affect the ESD results. In this study, a total of 1047 lesions in 873 patients were treated during the five-year period, averaging approximately 210 ESDs each year. This is considered to be a moderate to high patient volume. Many experiences with cases and a relatively high patient volume are also needed for the improvement of ESD skills and were found to be important for safely performing ESD.

Two patients with cancer of the esophagus, 62 patients with cancer of the stomach and two patients with cancer of the colon refused additional treatment/surgery in spite of a non-curative resection in this study. The reasons for refusing additional treatment/surgery were varied, including advanced age, complicated diseases, *etc*. The long-term outcomes for such non-curative ESD patients who refused additional treatment/surgery may be of interest. It also may be important data for deciding on the indications for the treatment of elderly patients. However, we did not analyze the overall data in terms of the long-term outcomes for such non-curative ESD patients in this study, so a future study will be needed to clarify these issues.

In conclusion, ESD is associated with a few problematic complications. However, in this study, all complications were resolved using non-surgical therapies such as endoscopic clipping. ESD can be safely performed in clinics with established therapeutic methods and medical services to address potential complications.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is a minimally invasive treatment for gastrointestinal cancer. It is usually performed in general hospitals because of the high frequency of complications and the need for a high level of technical skill. The aim of this study was to determine whether ESD can be safely performed at small clinics. The efficacy, technical feasibility and associated complications of the procedures were assessed.

Research frontiers

ESD is usually performed in general hospitals. Treatment data and outcome of ESD at general hospitals were reported. However, such reports from clinics were rare. The efficacy, technical feasibility and associated complications of the ESD procedures at a local clinic were assessed.

Innovations and breakthroughs

This study showed that ESD can be safely performed in clinics with established therapeutic methods and medical services to address potential complications. ESD is associated with a few problematic complications. However, in this study, all complications were resolved using non-surgical therapies such as endoscopic clipping.

Applications

ESD can be safely performed in clinics with established therapeutic methods and medical services to address potential complications.

Terminology

ESD is a minimally invasive treatment for gastrointestinal cancer. One-piece resection is considered the gold standard for endoscopic treatment because it provides an accurate histological assessment and reduces the risk of recurrence. ESD enable us to resect large and ulcerative lesions *en bloc*.

Peer review

This study investigated whether ESD can be safely performed at small clinics. The authors indicated ESD can be safely performed in their small 19-bed clinic with similar ESD results as previously published from general hospitals. This is an interesting topic.

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BRIEF ARTICLE

Cdx2 expression and its promoter methylation during metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus

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Abstract

AIM: To examine how the expression of caudal type homebox transcription factor 2 (Cdx2) is regulated in the development of malignancy in Barrett's esophagus.

METHODS: Cdx2, mucin (MUC) series (MUC2, MUC5AC and MUC6), p53 and E-cadherin expression in Barrett's esophagus and adenocarcinoma specimens were examined by immunostaining. Isolated clusters of cells from (1) MUC2 and Cdx2-positive intestinal metaplastic mucosa; (2) MUC5AC and MUC6-positive, and MUC2 and Cdx2-negative high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC); and (3) MUC5AC, MUC6 and Cdx2-positive poorly-differentiated invasive adenocarcinoma (PDA) were analyzed by methylation-specific polymerase chain reaction using sets of primers for detecting methylation status of the *Cdx2* gene.

RESULTS: Most of the non-neoplastic Barrett's esophageal mucosa showing intestinal-type metaplasia with or without low-grade dysplasia was positive for E-cadherin, MUC series and Cdx2, but negative for p53. A portion of the low-grade to HD was positive for E-cadherin, MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. The definite IMC area was strongly positive for MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. Methylation of the Cdx2 promoter was not observed in intestinal metaplasia, while hypermethylation of part of its promoter was observed in hot dipped and IMC. Hypermethylation of a large fraction of the Cdx2 promoter was observed in PDA.

CONCLUSION: Cdx2 expression is restored irrespective of the methylation status of its promoter. Apparent positive immunohistochemical results can be a molecular mark for gene silencing memory.

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Key words: Barretti's esophagus; Caudal type homebox transcription factor 2; Intestinal metaplasia; Promoter hypermethylation

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INTRODUCTION

Barrett's esophagus, first described in 1950 and refined in 1957, is a condition whereby the distal esophageal squamous epithelium is replaced by metaplastic columnar epithelium[1]. Three types of morphologically distinct

metaplastic columnar epithelia are recognized in Barrett's esophagus: gastric-fundic, gastric-cardiac (junctional type), and intestinal (specialized type) metaplasia[2]. Reflecting a finding that patients with intestinal-type epithelium are at increased risk of developing adenocarcinoma, the American College of Gastroenterology has recently proposed a restricted definition of Barrett's esophagus: "a change in the esophageal epithelium of any length that can be recognized at endoscopy and is confirmed to have intestinal metaplasia at biopsy"[3]. Although a recent cohort study has demonstrated that the frequency of cancer development in Barrett's esophagus is not related to the presence of intestinal metaplasia, metaplastic columnar epithelium, *per se*, is generally accepted as a precancerous process predisposed to develop discrete neoplastic lesions such as the gastric or foveolar type, the adenomatous or intestinal type, hybrid type dysplasia, and intramucosal [high-grade dysplasia (HD) or intramucosal adenocarcinoma (IMC)] and invasive cancers[4]. Cancers derived from Barrett's esophagus are histopathologically classified into two major categories: gastric and intestinal[5]. Since most Barrett-related IMC cases are either gastric or intestinal with distinct phenotypic stability during progression, two separate (gastric and intestinal) pathways of carcinogenesis have been proposed[5]. Importantly, during the progression of the intestinal pathway, a gradual decrease in transcription factor caudal type homebox transcription factor 2 (Cdx2, a caudal-related homeobox gene essential for skeletal and intestinal development has been noted, suggesting its tumor suppressor role in Barrett's esophagus[5].

We encountered a case of invasive esophageal adenocarcinoma developing into intestinal-type dysplasia and IMC, and examined Cdx2 expression and its promoter methylation status in close histopatho- logical relation to the progression stages with the use of microdissection and methylation-specific polymerase chain reaction (MSP).

MATERIALS AND METHODS

Patient

An 81-year-old Japanese man was admitted to our hospital complaining of heartburn especially after eating sweet fare. The patient had undergone stomach surgery (distal partial gastrectomy) due to gastric ulcer nearly forty years earlier. Because of gastric regurgitation, he had undergone endoscopic examination of the upper digestive tract, which revealed severe reflux esophagitis with widespread Barrett's esophagus. A biopsy was taken from irregularly elevated lesions inside the Barrett's esophagus, and a histological examination confirmed esophageal adenocarcinoma in the lesions. An esophagectomy was carried out, and the right hemicolon was rebuilt. The patient has been free of recurrence for two years since the operation.

Immunohistochemistry

The specimens of Barrett's esophagus were subjected to immunohistochemistry using diaminobenzidine as the chromogen. Deparaffinized sections of formalin-fixed tissue were stained with mucin (MUC) series (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), p53 (Lab Vision, Kalamazoo, United States) and E-cadherin (Dako, Denmark) antibody diluted at 1:100 after heat-induced antigen retrieval and with Cdx2 (Dako, Denmark) antibody diluted at 1:50. Anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody for p53 and anti-mouse IgG was used for MUC series, Cdx2 and E-cadherin.

Agarose-bead mediated template preparation

Paraffin-embedded samples were deparaffinized in xylene and subjected to microdissection under light microscopic observation (Leica Microsystems, LMD7000) with the aid of both E-cadherin immunostaining and Cdx2 immunostaining. The microdissected samples were liquefied in low-melting agarose (3.2%) at 1:1, and agarose beads were made by chilling on ice. Beads were treated with proteinase K, followed by bisulfite conversion, as previously described[6].

Polymerase chain reaction amplification and sequencing

Bead fragments were analyzed by MSP using sets of primers for accessing the methylation status of the *Cdx2* gene. The promoter region of the human *Cdx2* genomic sequence (GenBank accession no. AL591024) was searched for CpG islands with an online search engine (www.ebi.ac.uk/emboss/cpgplot). One of the CpG islands (AL591024 nt 28391-28683) was further analyzed for methylation status by MSP. In the first-step polymerase chain reaction (PCR) amplification, a 183-bp amplicon containing 71-bp CpG sites, was amplified with two primers, (forward) 5'-GCCAAGGGGCCTAGGGCTGGA-3', and (reverse) 5'-GTTCACCTCCTAATACAAGCCTTTG-3' (Table 1), under the following conditions: 98°C 2 min, 30 cycles (98 ℃ 10 s, 50 ℃ 15 s, 68 ℃ 39 s). The primers used for second-step PCR were, (forward) 5'-GGAGCTGCCCCGACAGGAGCG-3', and (reverse) 5'-CGCGCCCAGCTCGGn TTTCAGCAA-3' (Table 1), under the following conditions: 98 ℃ 2 min, 25 cycles (98 ℃ 10 s, 60 ℃ 15 s, 68 ℃ 30 s). The PCR mixture contained Mighty AMP® DNA polymerase (Takara, Tokyo, Japan) and bead fragments in a final volume of 25 μL. The PCR products were electrophoresed in a 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

Ethics

Written informed consent was obtained from this patient, and this study was reviewed and approved by the local ethics committee at Ehime University.

RESULTS

Pathological findings

Grossly, a superficial spreading IMC surrounded by low-

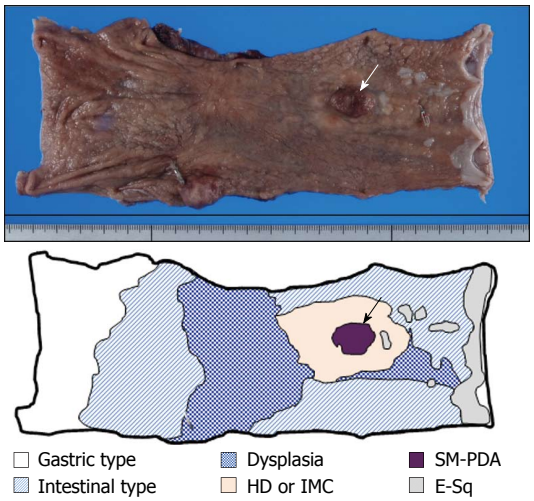

Figure 1 Macroscopic findings of excised esophagus. Surgical specimen shows the presence of superficial spreading carcinoma, extending between 30 mm from the oral and 105 mm from the anal surgical margins. The superficial spreading region is mostly composed of high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC), surrounded by dysplastic change (E-sq low-grade dysplasia). Inside this superficial spreading region, one observable elevated nodule (arrow) is composed of solid and submucosal invasive poorly-differentiated invasive adenocarcinoma (SM-PDA) with lymphatic invasion. The background non-neoplastic esophageal mucosa is extensively replaced by glandular mucosa with and without intestinal metaplasia.

Table 1 Primer sequences used in polymerase chain-based assays, product size and annealing temperature

	Primer sequence	Size (bp)	Temp (℃)
First PCR	(F)5′-GTTAAGGGGTTTAGGGTTGGA	183	60
Nested PCR	(R)5′-CAAAAACTTATATTAAAAAATAAAC		
Methylated	(F) 5′-GGAGTTGTTTCGATAGGAGCGC	71	60
	(R) 5′-TTACTAAAACCGAACTAAACGCG		
Unmethylated	(F) 5′-GGAGTTGTTTTGATAGGAGTGT	71	60
	(R) 5′-TTACTAAAACCAAACTAAACACA		

Temp: Temperature; PCR: Polymerase chain reaction; R: Reverse; F: Forward.

Table 2 Summary of immunohistochemical findings

	G-type	I-type (IM)	HD or IMC	PDA
MUC2	-	+	-	-
MUC5AC	+	-	++	+
MUC6	+	-	++	+
p53	-	-	++	++
E-cadherin	++	++	++	+
Cdx2	-	+	-	+

G-type: Gastric metaplasia; IM: Intestinal metaplasia; HD or IMC: High-grade dysplasia or intramucosal adenocarcinoma; PDA: Poorly-differentiated invasive adenocarcinoma; MUC: Mucin.

grade dysplasia and intestinal-type metaplasia extended between 30 mm from the oral and 105 mm from the anal surgical margins (Figure 1). One elevated nodule was noted inside this superficial spreading region (Figure 1). Microscopically, the background non-neoplastic esophageal mucosa was replaced, very extensively, by gastric foveolar type mucosa with (Figure 1) and without intestinal metaplasia (Figure 1). The superficial spreading IMC region was mostly composed of definite well-differentiated tubular adenocarcinoma or HD, surrounded by dysplastic change (low-grade dysplasia). The oral elevated nodular ridge was a solid, poorly-differentiated, invasive adenocarcinoma with lymphatic invasion, but no venous invasion or metastasis within the esophageal mucosa was observed. Immunohistochemically, the original esophageal squamous epithelium was positive for E-cadherin, but negative for all the MUC series (MUC2, MUC5AC and MUC6), Cdx2 and p53. Most of the non-neoplastic Barrett's esophageal mucosa showing intestinal-type metaplasia with or without low-grade dysplasia was positive for E-cadherin, MUC2, MUC5AC, MUC6 and Cdx2, but negative for p53. A portion of the low-grade to high-grade dysplasia was positive for E-cadherin, MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. The definite IMC area was strongly positive for MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. Figure 2 shows the transitional area between the intestinal metaplasia with low-grade dysplasia and the definite IMC area. A portion of the poorly-differentiated adenocarcinoma was positive for MUC5AC, MUC6, Cdx2 and p53, but negative for MUC2 (Figure 3). Table 2 shows a summary of the immunohistochemical findings.

Microdissection and MSP of the Cdx2 promoter

MSP revealed no methylation in Cdx2-positive Barrett's mucosa with intestinal metaplasia (Figure 4, intestinal type). Microdissected samples from the Cdx2-negative IMC area showed that a fraction of the cells was hypermethylated (Figure 4, IMC). Although Cdx2 expression was found by immunohistochemical analysis, samples from the poorly-differentiated invasive (PDA) area showed a hypermethylation pattern (Figure 4, submucosal invasive PDA).

DISCUSSION

Cdx2 is an intestine-specific transcription factor expressed in cells constituting the mucosal epithelium from the duodenum to the rectum[7]. While Cdx2 is negative for the normal foveolar mucosa of the stomach and the squamous epithelium in the esophagus stemming from the foregut, its heterotopic expression in Barrett's esophagus is observed especially in cases with intestinal-type metaplasia[8]. Among most of the terminal differentiation-specific transcription factors, Cdx2 is known to play a tumor suppressor role in cancer progression in the distal colon, a role, which in adults, is functionally and geographically distinct from the homeotic role of Cdx2 during gut development[9]. In our present case, and in confirming its tumor suppressor role, Cdx2 expression diminished during the progression from intestinal-type metaplasia to distinct IMC. Mirroring Cdx2 expression at the protein level by immunohistochemistry, the hypermethylation of the *Cdx2* gene promoter was revealed (Figure 4, IMC). Since primers used for MSP are set to amplify the *Cdx2* gene promoter with hypermethylation,

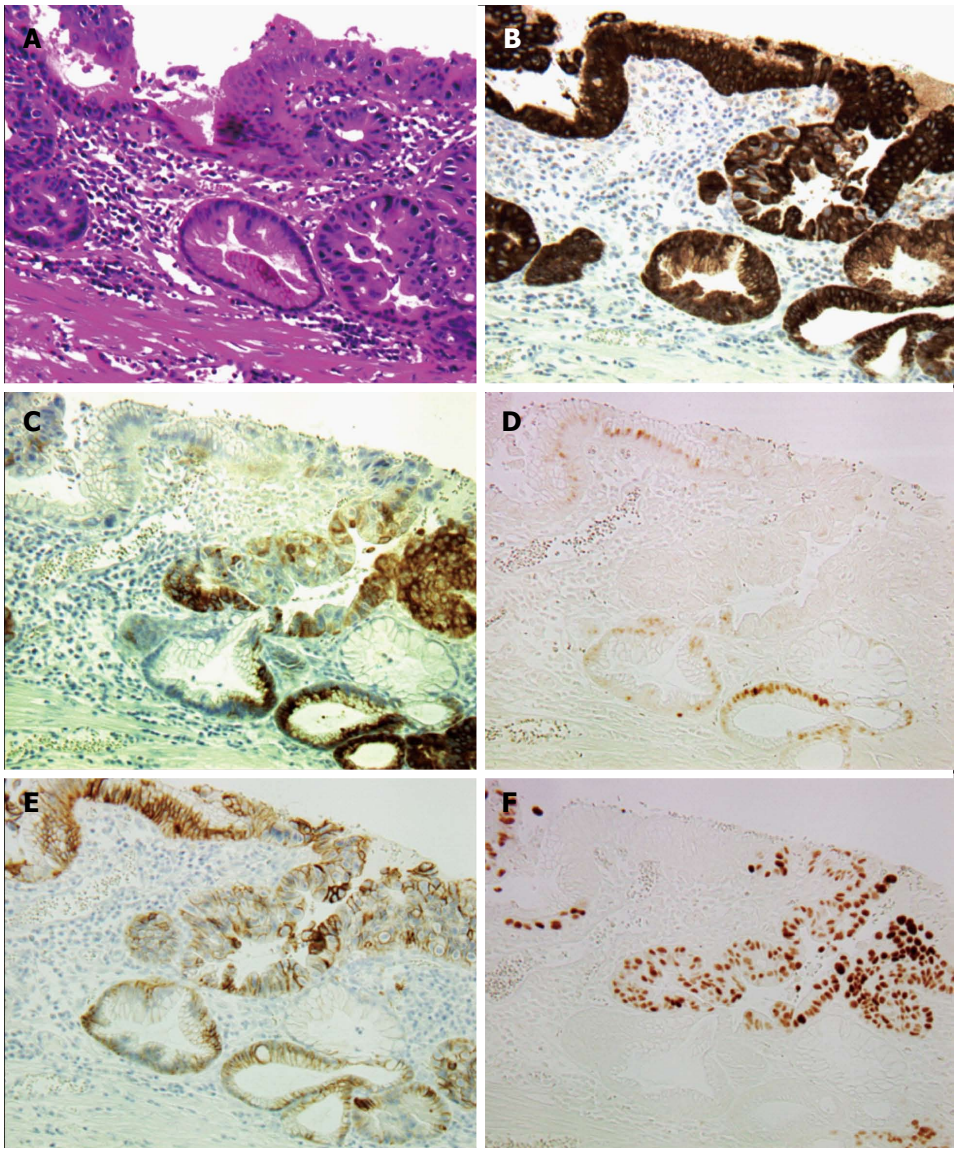

Figure 2 Histological findings of transitional area between intestinal metaplasia and high-grade dysplasia or intramucosal adenocarcinoma (× 200). A: Hematoxylin and eosin staining of transitional area. Intestinal metaplasia (IM) stretches from the upper left to the lower right corner; B: Mucin (MUC) 5AC immunostaining. Strong MUC5AC expression is observed in both IM and high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC) areas; C: MUC6 immunostaining. MUC6 expression is observed mostly in parts of the HD or IMC areas; D: Caudal type homebox transcription factor 2 (Cdx2) immunostaining. Cdx2 expression is observed only in the nuclei of the cells in the IM area; E: E-cad immunostaining. E-cad expression is observed on the membranes of cells in both IM and HD or IMC areas; F: p53 immunostaining. Strong p53 expression is observed in the nuclei of the cells in the HD or IMC area.

i.e., when all CpGs are methylated, a large fraction of the cells may acquire partial or scatter-type CpG methylation and, therefore, the *Cdx2* gene promoter may have been underestimated in our MSP. In support of our current study, Khor *et al*[5] also demonstrated the gradual down-regulation of Cdx2 expression during progression in adenomatous dysplasia, at least in the intestinal pathway of the Barrett esophageal cancers. These data suggest that Cdx2 also plays a tumor-suppressor role in the metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus. In our present case, irrespective of the hypermethylation status of the *Cdx2* gene promoter, Cdx2 expression was restored in PDA as analyzed by immunohistochemistry (Figure 3F). To achieve final gene-silencing, chromatin condensation followed by modifications of histone proteins are essential[10], we therefore hypothesize that epigenetic alterations other than demethylation may lead to *Cdx2* gene reactivation during the progression phase. Indeed, our previous study showed that hypermethylation of the *E-cadherin* gene promoter and MeCP2, a methyl-CpG binding domain protein, synergistically silenced gene expression in colorectal cancers[6]. Therefore, it is evident that hypermethylation of the gene promoter, *per se*, is essential for establishing gene silencing, but not sufficient for blocking gene expression. Since in our present case, Cdx2 reactivation did not correlate with differentiated intestinal phenotype, but was observed in invasive or aggressive phenotypes, the tumor suppressive effect of Cdx2 on these invasive cancer cells might be lost. These somewhat complicated epigenetical events may partly explain the dispersion of Cdx2 expression. Therefore, when characterizing cancer cells by immunophe-

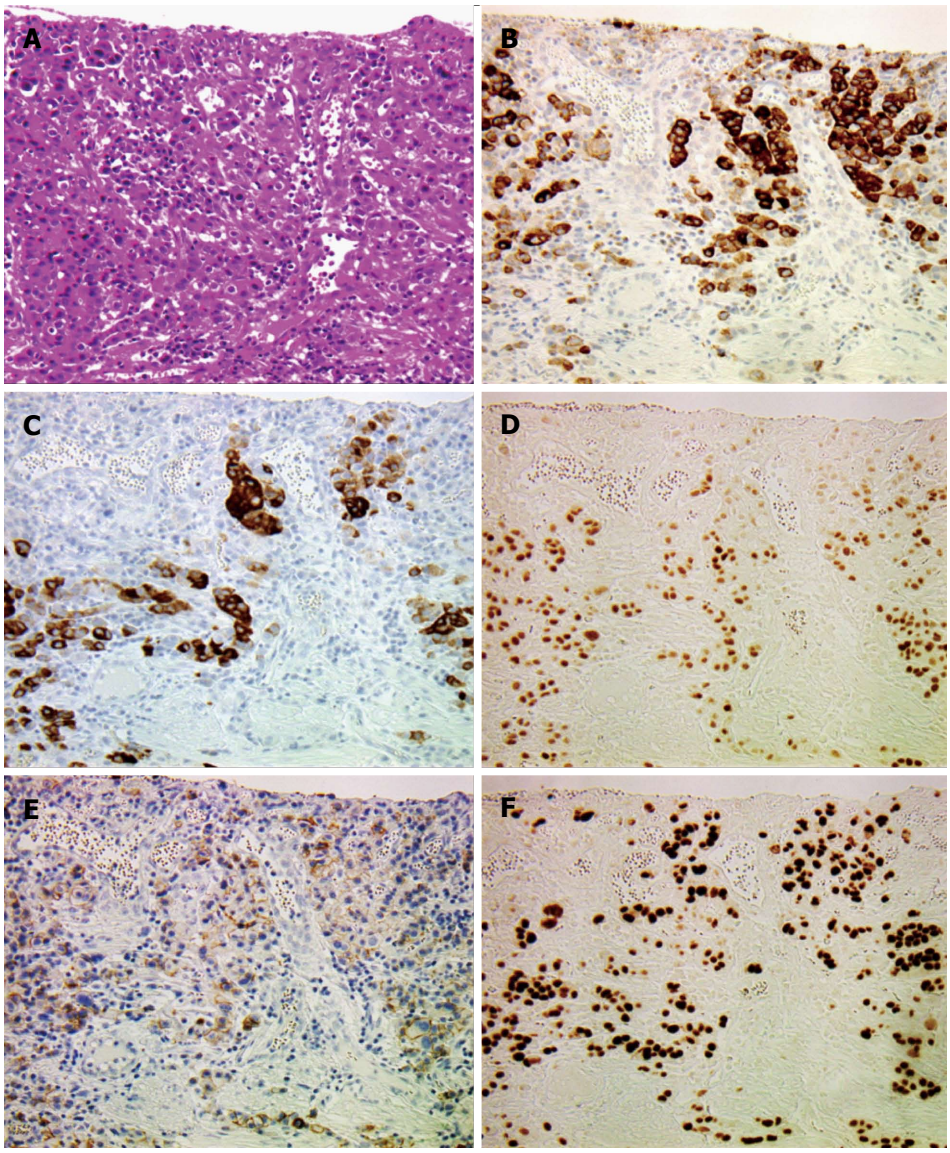

Figure 3 Histological findings of diffuse poorly-differentiated invasive adenocarcinoma (× 200). A: Hematoxylin and eosin staining of poorly-differentiated invasive adenocarcinoma (PDA). Cancer cells are diffusely scattered with prominent stromal reaction; B: Mucin (MUC) 5AC; C: MUC6; D: Caudal type homebox transcription factor 2 expression is positive in the PDA area; E: E-cad expression is markedly reduced in the PDA area; F: Strong p53 expression is observed in the nuclei of the cells in the PDA area.

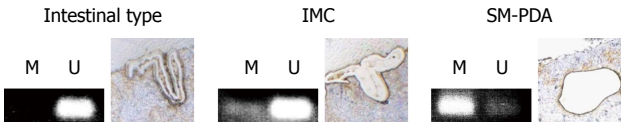

Figure 4 Detection of methylated cytosine by methylation-specific polymerase chain reaction analysis of caudal type homebox transcription factor 2 CpG-island region. Tissue samples were stained with E-cadherin and caudal type homebox transcription factor 2 (Cdx2) to assist cell identification. Cells, either isolated from intestinal metaplasia, intramucosal adenocarcinoma (IMC) or submucosal invasive poorly-differentiated adenocarcinoma (SM-PDA) by laser-assisted microdissection, were subjected to bisulfite conversion and subsequent methylation-specific polymerase chain reaction (MSP). MSP products using primers that specifically amplify only unmethylated DNA are indicated by visible polymerase chain reaction products in line unmethylated pattern (U), while visible polymerase chain reaction products in line methylated pattern (M) indicate those amplified by primers specific for methylated DNA. In intestinal-type metaplasia sections, MSP shows a U pattern, while MSP shows U patterns with partial M patterns in high-grade dysplasia or IMC sections. MSP shows a mostly M pattern in PDA where strong Cdx2 expression is observed (Figure 3D).

notyping, any apparent positive immunohistochemical results should be interpreted carefully with the help of the hypermethylation status as a molecular mark for gene silencing memory[10,11].

ACKNOWLEDGMENTS

We thank Ms. Yuki Takaoka for technical assistance.

COMMENTS

Background

Barrett's esophagus, a pathological condition in which the esophageal squamous epithelium is replaced by metaplastic columnar mucosa, is known to predispose to the development of dysplasia and subsequent cancers.

Research frontiers

Caudal type homebox transcription factor 2 (Cdx2) has recently been shown to play a tumor-suppressor role in the 'metaplasia-dysplasia-carcinoma sequence'

in Barrett's esophagus.

Innovations and breakthroughs

Recent reports have evaluated the phenotypic stability and role of Cdx2 in the neoplastic progression of different types of dysplasias. This suggests that non-intestinalized columnar metaplasia may be an unstable intermediate state at risk for neoplastic progression.

Applications

When characterizing cancer cells by immunophenotyping, any apparent positive immunohistochemical results should be interpreted carefully with the help of the hypermethylation status as a molecular mark for gene silencing memory.

Peer review

The authors examined the expression of Cdx2 and its methylation in Barrett metaplasia and esophageal adenocarcinoma. It revealed that irrespective of the hypermethylation status of the *Cdx2* gene promoter, Cdx2 expression was restored in poorly-differentiated invasive adenocarcinoma. The results are interesting and when characterizing cancer cells by immunophenotyping, any apparent positive immunohistochemical result should be interpreted carefully.

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BRIEF ARTICLE

Relationship between diversion colitis and quality of life in rectal cancer

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Abstract

AIM: To investigated the incidence of diversion colitis (DC) and impact of DC symptoms on quality of life (QoL) after ileostomy reversal in rectal cancer.

METHODS: We performed a prospective study with 30 patients who underwent low anterior resection and the creation of a temporary ileostomy for the rectal cancer between January 2008 and July 2009 at the Department of Surgery, Korea University Anam Hospital. The participants totally underwent two rounds of the examinations. At first examination, endoscopies, tissue biopsies, and questionnaire survey about the symptom were performed 3-4 mo after the ileostomy creations. At second examination, endoscopies, tissue biopsies, and questionnaire survey about the symptom and QoL were performed 5-6 mo after the ileostomy reversals. Clinicopathological data were based on the histopathological reports and clinical records of the patients.

RESULTS: At the first examination, all of the patients presented with inflammation, which was mild in 15 (50%) patients, moderate in 11 (36.7%) and severe in 4 (13.3%) by endoscopy and mild in 14 (46.7%) and moderate in 16 (53.3%) by histology. At the second examination, only 11 (36.7%) and 17 (56.7%) patients had mild inflammation by endoscopy and histology, respectively. There was no significant difference in DC grade between the endoscopic and the histological findings at first or second examination. The symptoms detected on the first and second questionnaires were mucous discharge in 12 (40%) and 5 (17%) patients, bloody discharge in 5 (17%) and 3 (10%) patients, abdominal pain in 4 (13%) and 2 (7%) patients and tenesmus in 9 (30%) and 5 (17%) patients, respectively. We found no correlation between the endoscopic or histological findings and the symptoms such as mucous discharge, bleeding, abdominal pain and tenesmus in both time points. Diarrhea was detected in 9 patients at the second examination; this number correlated with the severity of DC (0%, 0%, 66.7%, 33.3% *vs* 0%, 71.4%, 23.8%, 4.8%, $P = 0.001$) and the symptom-related QoL ($r = -0.791$, $P < 0.001$).

CONCLUSION: The severity of DC is related to diarrhea after an ileostomy reversal and may adversely affect QoL.

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Key words: Diversion colitis; Quality of life; Diarrhea; Ileostomy; Rectal cancer

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INTRODUCTION

The surgical interruption of fecal flow may induce inflammation in the non-functional region of the distal colon[1]. This condition, diversion colitis (DC), is particularly common after an ileostomy or a colostomy. Theoretically, the inflammation typically resolves when the fecal passage resumes[2]. In Western countries, the estimated incidence of DC ranges from 70% to 100%[1,3], but the incidence in Asian countries is unknown. Glotzer *et al*[4] first described the clinical symptom related to defunctioned colon. DC symptoms include abdominal pain, bleeding, mucous discharge, and tenesmus; however, many patients do not present with definitive symptoms[5]. Retrospective and prospective studies report abnormal endoscopic and histological findings in most DC patients[3,6-8]. For patients having rectal surgery for cancer, the functional outcome and quality of life (QoL) are related to the length of the remnant rectum, the radiation therapy parameters and the extent of anastomotic leakage[9-11]. After ileostomy reversal, DC may also influence QoL, but the significance of this influence has not been reported. We hypothesized that the endoscopic and histological incidence of DC before and after ileostomy reversal in rectal cancer patients will correspond with patient's symptoms and impact QoL. Thus, the aims of this study are to determine the incidence of DC and its impact on QoL after ileostomy reversal.

MATERIALS AND METHODS

Forty-eight consecutive patients had surgery for rectal cancer and the creation of a temporary ileostomy at our institution between January 2008 and July 2009. Rectal cancer was defined as tumor 16 cm or less from the anal verge measured with a rigid rectosigmoidoscope (lower third, < 6 cm; middle third, 6-12 cm; upper third, 12-16 cm). The eligible patients were aged 18 years or older with, an American Society of Anesthesiologists class 1 to 3 and diagnosed with rectal cancer within 16 cm from the anal verge. The initial exclusion criteria included inflammatory bowel disease, such as Crohn's disease or ulcerative colitis, preoperative or postoperative radiation therapy and anastomotic leakage after rectal cancer surgery. The indications for chemoradiation prior to surgery were as follows: T4 lesion involvement below the peritoneal reflection, bulky tumor mass or lateral pelvic lymph node metastasis. The surgical indications for rectal cancer included clinical T1-3 lesions based on a pelvic computed tomography and an magnetic resonance imaging, irrespective of any locally lymph node metastasis the within mesorectal fascia. For the enrolled patients, the stoma closure was performed 3-4 mo after the first surgery to allow for 8 patients with stage Ⅲ or Ⅳ disease to complete intravenous chemotherapy. Each patient was hospitalized on 1 d before the ileostomy reversal. At this time, they underwent the first round of endoscopy, tissue biopsy and completed a questionnaire. A single endoscopist conducted the colonoscopies without an enema to ensure the collection of abnormal tissues. Random biopsies were also performed on the descending or transverse colon, even in case of normal colonoscopic findings. A single pathologist examined the tissues. Both the endoscopist and the pathologist were blinded to the patient's symptoms. The first DC symptom questionnaire was also performed during these 3 to 4 mo. Five to six months after the ileostomy reversal, the same endoscopist and pathologist conducted endoscopic and histological examinations to evaluate for complete resolution of DC. In the second questionnaire, we investigated symptom changes and related QoL changes. The endoscopic findings were scored according to edema, mucosal hemorrhage and contact bleeding. These features were scored individually from 0 to 3, 0 to 3, and 0 to 1, respectively, for a total score of 0 to 7. The total score was defined as mild (1 to 2), moderate (3 to 5), or severe (6 to 7). The histological findings were scored according to acute inflammation, chronic inflammation, eosinophilic infiltration, crypt architecture distortion, follicular lymphoid hyperplasia and crypt abscess. These features were scored from 0 to 1; 0 to 2; 0 to 2; 0 to 1; 0 to 1; and 0 to 1, respectively, for a total score of 0 to 8. The total score was further defined as mild (1 to 3), moderate (4 to 6), or severe (7 to 8). Both of the questionnaires investigated DC symptoms such as mucous discharge, bleeding, abdominal pain and tenesmus. However, we excluded fecal frequency, incontinence and the symptoms that are associated with intestinal obstruction (abdominal distension and pain, and emesis). We also excluded the bleeding from hemorrhoids and anal fissures. The QoL section of the questionnaire assessed symptomatic changes from before to after ileostomy reversal as follows: "much worse", "slightly worse", "similar", "slightly better", or "much better". The institutional review board of our institution approved this study and all of the subjects provided informed consent.

Statistical analysis

The data were represented as the median (minimum-maximum), as appropriate for quantitative variables and frequency (percentage) for qualitative variables. The χ^2 test and Fisher's exact test were used to compare between the endoscopic and histological findings about incidence rates of DC, and to test difference or relation with symptoms according to severity of DC and QoL. *P* values less than 0.05 were considered statistically significant. The calculations were performed using SPSS version 12.0 (SPSS Inc, Chicago, IL, United States).

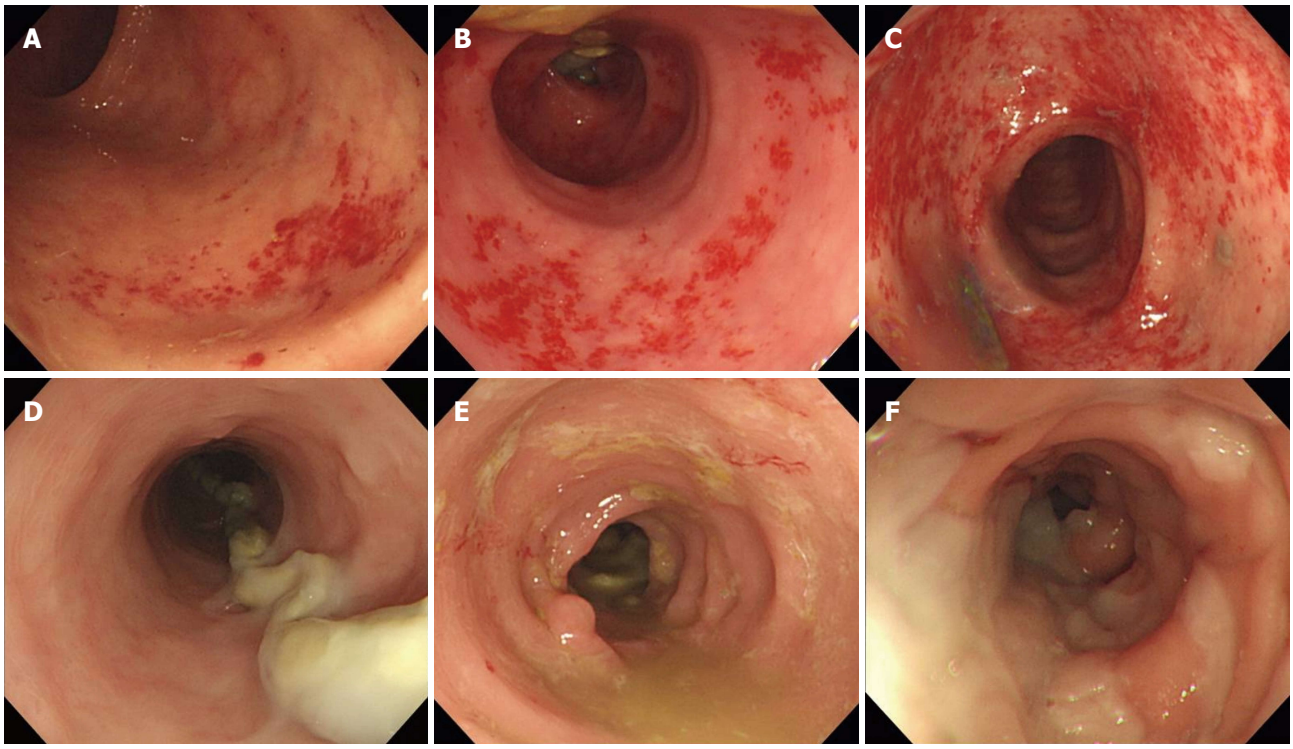

Figure 1 Example of major endoscopic findings in diversion colitis according to relative severity. A-C: Mucosal hemorrhage (A: Score 1; B: Score 2; C: Score3); D-F: Edema (D: Score 1; E: Score 2; F: Score 3).

Table 1 Characteristics for 30 patients with rectal cancer *n* (%)

Parameter	Patients
Age (yr)	59 (31-73)[1]
Gender	
Male	21 (70.0)
Female	9 (30.0)
ASA	
Ⅰ	11 (36.7)
Ⅱ	15 (50)
Ⅲ	4 (13.3)
Ⅳ	0
Tumor location	
Upper rectum	6 (20.0)
Mid rectum	5 (16.7)
Lower rectum	19 (63.3)
Intravenous chemotherapy	
No	22 (73.3)
Yes	8 (26.7)
pTNM staging	
0	4 (13.3)
Ⅰ	6 (20.0)
Ⅱ	11 (36.7)
Ⅲ	7 (23.3)
Ⅳ	2 (6.7)

[1]Values are median (range). ASA: American Society of Anesthesiologists; pTNM: Pathological tumor node metastasis.

RESULTS

Between January 2008 and July 2009, 48 patients were enrolled in the study. However, preceding the study, 18 patients were excluded from the analysis because they underwent a bowel enema procedure (7 patients) that could alter mucosal findings or symptoms, had excessive bowel content that hindered a clear colonoscopic view (9 patients), or had an anastomotic stricture observed during colonoscopy that prevented the visualization of the proximal portion of the colon (2 patients). The final prospective analysis included 30 patients.

The patients' characteristics are listed in Table 1. The median age was 59 years (range: 31-73 years) and the male: female ratio was 7:3. The tumor was located in the upper rectum for 6 patients (20%), the mid-rectum for 5 patients (16.7%) and the lower rectum for 19 patients (63.3%). Only 8 patients received intravenous chemotherapy. Using the endoscopic and histological criteria, all of the patients (100%) developed DC after their ileostomy creation (Table 2). There was no significant difference between the endoscopic and the histological findings in terms of the DC grade prior to ileostomy reversal ($P = 0.084$) or 5-6 mo after reversal ($P = 0.195$), and the endoscopic and histological features decreased after the ileostomy reversal. The incidence of symptomatic DC was 63.3% after ileostomy creation, and 46.6% 5-6 mo after ileostomy reversal. Figure 1 shows the endoscopic findings by the mucosal hemorrhage and edema severity. Figure 2 shows histological findings by the eosinophilic infiltration and chronic inflammation severity. Of the endoscopic findings, edema and mucosal hemorrhage were most common, and contact bleeding was rare. The most common histological finding was chronic inflammation (100%), followed by eosinophilic infiltration (Table 3). The endoscopic and histological abnormalities disappeared or decreased after the ileostomy reversal (Table 3).

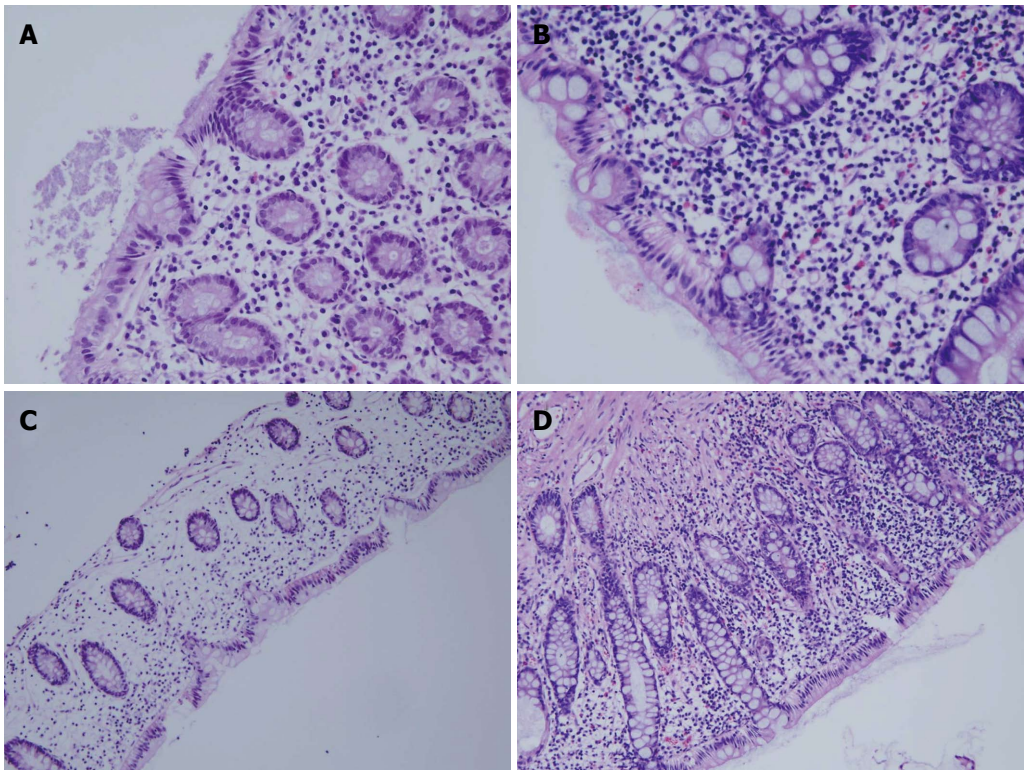

Figure 2 Example of major histological findings in diversion colitis according to relative severity. A, B: Eosinophilic infiltration including numerous cytoplasmic granules and bilobed nucleus (A: Score 1; B: Score 2; hematoxylin and eosin stain × 400); C, D: Chronic inflammation with mononuclear cell such as macrophage,lymaphocytes and plasma cells (C: Score 1; D: Score 2; hematoxylin and eosin stain × 200).

Table 2 Incidence of diversion colitis *n* (%)

Grade of DC	1 d prior to ileostomy reversal			5-6 mo after ileostomy reversal		
	Endoscopic (*n* = 30)	Histological (*n* = 30)	*P* value	Endoscopic (*n* = 30)	Histological (*n* = 30)	*P* value
None	0	0	0.084	19 (63.3)	13 (43.3)	0.195
Mild	15 (50.0)	14 (46.7)		11 (36.7)	17 (56.7)	
Moderate	11 (36.7)	16 (53.3)		0	0	
Severe	4 (13.3)	0		0	0	

DC: Diversion colitis.

Table 3 Endoscopic and histological findings *n* (%)

	1 d prior to ileostomy reversal (*n* = 30)	5-6 mo after ileostomy reversal (*n* = 30)
Endoscopic finding		
Edema	30 (100)	9 (30.0)
Mucosal hemorrhage	22 (73.3)	3 (10.0)
Contact bleeding	6 (20.0)	0
Histological finding		
Acute inflammation	7 (23.3)	0
Chronic inflammation	30 (100)	17 (56.7)
Eosinophilic infiltration	29 (96.7)	9 (30.0)
Crypt architecture distorsion	6 (20.0)	0
Follicular lymphoid hyperplasia	12 (40.0)	0
Crypt abscess	7 (23.3)	0

Before the ileostomy reversal, the most frequent initial symptom, according to the questionnaires, was mucous discharge, followed by tenesmus, bleeding and abdominal pain (Table 4). There were no reports of anal defecation caused by an ileostomy with incomplete diversion prior to the ileostomy reversal. The answers to the questionnaire conducted 5-6 mo after the ileostomy reversal showed that the symptoms had generally decreased since the first survey (Table 4). However, nine patients developed diarrhea as a new symptom (Table 4). We found no correlation between the endoscopic or histological findings and the symptoms before ileostomy reversal.

For example, there were mucous discharge ($P = 0.073$; $P = 0.284$), bleeding ($P = 0.338$; $P = 1.000$), abdominal pain ($P = 0.529$; $P = 0.602$), and tenesmus ($P = 0.075$; $P = 0.440$) (Table 4). Of the symptoms reported after the ileostomy reversal, only diarrhea correlated with the endoscopic and histological findings before the ileostomy reversal ($P = 0.001$; Table 4). In the second questionnaires, 4 patients indicated that they felt 'much worse', 8

Table 4 Correlation between symptoms before and after ileostomy reversal and grade of diversion colitis[1] *n* (%)

Symptom	Endoscopic finding (*n* = 30)				Histological finding (*n* = 30)			
	Mild	Moderate	Severe	*P* value	Mild	Moderate	Severe	*P* value
Symptoms before ileostomy reversal								
Mucous discharge				0.073				0.284
Yes	3 (25.0)	7 (58.3)	2 (16.7)		4 (33.3)	8 (66.7)	0	
No	12 (66.7)	4 (22.2)	2 (11.1)		10 (55.6)	8 (44.4)	0	
Bleeding				0.338				1.000
Yes	1 (20.0)	3 (60.0)	1 (20.0)		2 (40.0)	3 (60.0)	0	
No	14 (56.0)	8 (32.0)	3 (12.0)		12 (48.0)	13 (52.0)	0	
Abdominal pain				0.529				0.602
Yes	1 (25.0)	2 (50.0)	1 (25.0)		1 (25.0)	3 (75.0)	0	
No	14 (53.9)	9 (34.6)	3 (11.5)		13 (50.0)	13 (50.0)	0	
Tenesmus				0.075				0.440
Yes	2 (22.2)	6 (66.7)	1 (11.1)		3 (33.3)	6 (66.7)	0	
No	13 (61.9)	5 (23.8)	3 (14.3)		11 (52.4)	10 (47.6)	0	
Symptoms after ileostomy reversal								
Mucous discharge				0.679				0.336
Yes	3 (60.0)	1 (20.0)	1 (20.0)		1 (20.0)	4 (80.0)	0	
No	12 (48.0)	10 (40.0)	3 (12.0)		13 (52.0)	12 (48.0)	0	
Bleeding				0.550				0.228
Yes	1 (33.3)	1 (33.3)	1 (33.3)		0 (0)	3 (100)	0	
No	14 (51.9)	10 (37.0)	3 (11.1)		14 (51.9)	13 (48.1)	0	
Abdominal pain				0.229				0.485
Yes	1 (50.0)	0	1 (50.0)		0	2 (100)	0	
No	14 (53.8)	11 (39.3)	3 (10.7)		14 (50.0)	14 (50.0)	0	
Tenesmus				0.844				0.336
Yes	2 (40.0)	2 (40.0)	1 (20.0)		1 (20.0)	4 (80.0)	0	
No	13 (52.0)	9 (36.0)	3 (12.0)		13 (52.0)	12 (48.0)	0	
Diarrhea				0.001				0.001
Yes	0	6 (66.7)	3 (33.3)		0	9 (100)	0	
No	15 (71.4)	5 (23.8)	1 (4.8)		14 (66.7)	7 (33.3)	0	

[1]Grade of diversion colitis means result that we examined 1 d prior to ileostomy reversal.

Table 5 Correlation between symptoms and quality of life 5 to 6 mo after ileostomy reversal *n* (%)

Symptom	Much worse	Slightly worse	Similar	Slightly better	Much better	*P* value
Mucous discharge						0.137
Yes	2 (40.0)	0	2 (40.0)	1 (20.0)	0	
No	2 (8.0)	8 (32.0)	13 (52.0)	2 (8.0)	0	
Bleeding						0.797
Yes	0	1 (33.3)	2 (66.7)	0	0	
No	4 (14.8)	7 (25.9)	13 (48.1)	3 (11.1)	0	
Abdominal pain						0.816
Yes	0	1 (50.0)	1 (50.0)	0	0	
No	4 (14.3)	7 (25.0)	14 (50.0)	3 (10.7)	0	
Tenesmus						0.516
Yes	1 (20.0)	2 (40.0)	1 (20.0)	1 (20.0)	0	
No	3 (12.0)	6 (24.0)	14 (56.0)	2 (8.0)	0	
Diarrhea						< 0.001[1]
Yes	4 (44.4)	5 (55.6)	0	0	0	
No	0	3 (14.3)	15 (71.4)	3 (14.3)	0	

[1]Spearman's correlation coefficient = -0.791.

patients felt 'slightly worse', 15 patients felt "similar" and 3 patients felt "slightly better" (Table 5). Finally, diarrhea showed a significant effect in the QoL relevance analysis of symptoms ($r = -0.791$, $P < 0.001$; Table 5).

DISCUSSION

Both the endoscopic and histological findings showed that 100% of the patients developed DC following the ileostomy creation. This figure exceeds the rates reported in Western populations (91% and 76%)[5,8]. The symptoms previously associated with DC include mucous discharge, rectal bleeding, abdominal pain and tenesmus[5]. We can check for diarrhea in patients with normal bowel continuity of bowel, but in patients with an ileostomy, we cannot. We expected that diarrhea would occur in DC after the ileostomy reversal as it commonly occurs in other forms of colitis (ulcerative colitis, Crohn's colitis and ischemic colitis). We tested this hypothesis using a questionnaire concerning symptoms, including diarrhea, with a study group that excluded the patients with inflammatory bowel disease and ischemic colitis.

DC symptom rates vary from 6% to 48%, although the overall occurrence is low[5,12,13]. Nineteen patients (63.3%) in our study had at least one symptom after ileostomy creation, and the incidence of symptoms after the ileostomy reversal was 46.6%. Thus, the incidence appears to have decreased slightly; however, these rates represent a dynamic situation. The current analysis showed that mucous discharge, bleeding, abdominal pain and tenesmus resolved, in 11 of 12 patients, 4 of 5 patients, all of 4 patients and 6 of 9 patients, respectively, after the

ileostomy reversal. Most of the symptoms disappeared; however, the symptoms experienced after the ileostomy reversal were either were continued or new but likely not caused by DC. For example, after the ileostomy reversal, 6 patients reported tenesmus; it was a new symptom in 3 patients and continued in 3 patients. Tenesmus can occur as the result of the rectal resection itself. Some authors have suggested that tenesmus resulted from altered motility and the reservoir function of the neorectum[14]. Considering the lack of a relationship between the DC symptoms (except diarrhea), and DC severity in this study, we assume that it is difficult to judge DC severity by these symptoms, particularly in patients with a temporary ileostomy. The endoscopic and histological findings, indicate that the inflammatory changes in DC resolve quickly after intestinal continuity is reestablished[5]. Some authors report normal sigmoidoscopy within 2 mo after restoring colonic continuity[4]. The patients in our study still had mild DC 5-6 mo after the ileostomy reversal. Therefore, the time to complete resolution of DC may vary and may not be predictable. To confirm this hypothesis, further evaluation is needed.

We must consider more active treatment selectively in symptomatic patients who have severe endoscopic/histological findings. There are two options for treating of symptomatic DC. DC symptoms may persist in patients with a permanent stoma. One patient with hemiplegia, for whom stoma reversal was not feasible, underwent rectal resection for severe DC[15]. Surgical treatment may have been appropriate here, but other studies show that medical treatment may also be effective. Early studies suggested that a deficiency of short chain fatty acids (SCFAs) in the excluded colon would contributes to DC[1]. Bacteria produce SCFAs as byproducts of carbohydrate fermentation in the colonic lumen, and SCFAs provide the primary energy source for colonic mucosal cells[15]. Some authors even argue that SCFAs enema may improve the symptoms and endoscopic findings[1]. In human neutrophils, SCFAs reduce the production of reactive oxygen species, which are the agents of oxidative tissue damage[16]. Therefore, SCFAs may be applicable to symptomatic DC patients who have temporary ileostomies.

The current study showed that there were no severe DC symptoms in the patients who had temporary ileostomies. This finding explains why the patients easily endured the ileostomy or, even though they were developed, and were able to wait for timing of ileostomy reversal after clinician's explanation. Most clinicians can predict which symptoms, may not merit treatment clinically.

Patients may not demand active treatment for other symptoms, but they will for diarrhea. The diarrhea appeared soon after the ileostomy reversal, and continued for a minimum of 3 wk and a maximum of 8 wk in this study. Most clinicians previously thought of diarrhea as low anterior resection syndrome (LARS) and others mistook it for a symptom of general enterocolitis. Most of the patients complained of anal skin irritation, lethargy and nervousness. Some went so far as to continue with a stoma rather than have their ileostomy reversed. In the present study, the patients who had diarrheal symptoms and no signs of obstruction could obtain some degree of control with anti-diarrheal agents such as loperamide.

Several factors may affect functional outcome and in QoL of rectal cancer. Hoerske *et al*[11] reported that patients diagnosed with lower rectal cancer have lower QoL score than those with upper and middle rectal cancer because of the smaller reservoir volume. Low anterior resection (LAR) for rectal cancer can leads to severe bowel dysfunction with fecal frequency, urgency and incontinence[17-19]. Other evidence suggests that patients who receive preoperative radiotherapy have a higher rate of urgency, diarrhea and fecal incontinence[20,21]. Therefore, we excluded the patients who received preoperative radiotherapy to limit the confusion with DC symptoms. Generally, radiation therapy can be used as local treatment for rectal cancer. Simunovic *et al*[22] reported that the local recurrence rate of rectal cancer was 6% in a preoperative radiation group *vs* 3% in a non-radiation group. This rate may indicate that radiation therapy can be omitted if surgery is performed on the proper surgical plane with exact preoperative evaluation of the mesorectal fascia. In this study, most of the patients with locally advanced cancer already had lymph node metastases within the mesorectal fascia in the preoperative evaluation. In the United States, however, postoperative chemoradiation therapy has been the standard treatment because of improved local control, without any effect on the overall survival rate[23]. In this study, the patients an advanced cancer stage, underwent chemotherapy only. Hypoxia in the post-surgical bed requires more radiation dose than preoperative state. Postoperative radiation can increase the possibility of acute and chronic gastrointestinal toxicities, such as radiation enterocolitis (including proctitis) and adhesions affecting the small bowel[24]. These events can negatively affect a patient's QoL. Postoperative complications such as anastomotic leakage after LAR can also result in poor long-term functional outcomes[9].

Although some important factors in functional outcome or QoL in this context are known, the role of DC after restoration of fecal movement is unknown. We conducted this study for this reason. The nine patients who developed diarrhea after the ileostomy reversal subsequently reported a moderate to large decrease in QoL. Most of the other symptoms did not correlate with the QoL. Diarrhea was also the only symptom that showed a significant correlation with the severity of DC; hence, we suggest that severe DC can be predicted by the occurrence of diarrhea alone after an ileostomy reversal. In the clinical environment, we propose that clinicians continue treatment with SCFA enemas instead of ileostomy reversal in patients with severe DC.

There are a few limitations in this study. First, the small sample size limited the statistical significance of our findings. Second, we did not use a validated questionnaire,

such as the Fecal Incontinence Quality of Life score analyzing multidimensional aspects that can indirectly reflect QoL. However, considering that the effectiveness of a questionnaire as a data collection tool depends on the nature of the study and the age of patients, we believe that modification of the questionnaire is needed. Third, 25 patients developed LARS with fecal frequency, urgency or incontinence after rectal cancer surgery. During the survey, most patients mistook fecal frequency or urgency for diarrheal symptom. Diarrhea is generally defined as the passage of abnormally liquid stool at an increased frequency, exceeding 200 g/d[25]. Although we tried to survey using an accurate definition, it was difficult to measure the total amount of stool per day. Future studies with a larger sample size, such as a multi-variate analysis of risk factors will be required to ascertain the relationship between LARS and diarrheal symptom. Finally, the pathogenesis of DC has been little reported until now. Some authors suggested that nitrate-reducing bacteria was found to be increased in the colon that is not receiving fecal flow[26]. We are investigating this issue at our hospital based on the hypothesis that differences in the colonic bacterial spectra may play a role in this variation.

In conclusion, in this study of South Korean patients diagnosed with rectal cancer, we observed a higher incidence of DC after temporary ileostomy than that reported for patients in Western countries. This is also the first study to indicate that DC after a temporary ileostomy may adversely affect patient QoL. The severity of DC after the closure of a diverting stoma appeared to influence patient QoL, primarily by causing diarrhea.

COMMENTS

Background

Up to now, the incidence of diversion colitis (DC) in western countries has been published in English literature, but not yet in eastern. Theorectically, DC can happen to patients with ileostomy after low anterior resection (LAR) in rectal cancer, in which patient's quality of life (QoL) after rectal resection is not only related to the remnant rectal length, but can be hypothesized to related to DC.

Research frontiers

Studies regarding the DC have mainly been limited to analyze clinicopathologic finding after surgical situation of the benign disease. Therefore, in patients with temporary ileostomy after LAR in rectal cancer, it would be great interest to examine the DC incidence in Asia and to evaluate if the severity of DC is related to patient's QoL after the ileostomy reversal.

Innovations and breakthroughs

The results of this study showed that DC developed in all patients with ileostomy after LAR and the symptoms in the period of diverting stoma were not correlated with severity of DC. But, diarrhea after the ileostomy reversal was related to the severity of DC.

Applications

There is a need to take notice of DC effect on the patient's QoL in rectal cancer. Severity of DC before an ileostomy reversal in rectal cancer can predict the patient's QoL after an ileostomy reversal and lead the patients into proper education and more intensive management. Also, on the base of this result, clinicians need to proceed the further study to clarify why the severity of DC is variable between the individual with patient's symptoms and impact QoL.

Terminology

DC is defined as the surgical interruption of fecal flow induce inflammation in the non-functional region of the distal colon and this is characterized that the inflammation typically can resolve when the fecal passage resumes.

Peer review

This paper is very well written in spite of a small sample size and has a possible message that the severity of DC seems to have an influence on patient's QoL, particularly on diarrhea, after reversal of a diverting stoma.

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BRIEF ARTICLE

Colon transit time according to physical activity and characteristics in South Korean adults

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Abstract

AIM: To investigate factors contributing to the colon transit time (CTT), physical activity and characteristics were examined.

METHODS: Forty-seven Korean adults (males, *n* = 23; females, *n* = 24) took a capsule containing 20 radio-opaque markers to measure the CTT. The subjects used an accelerometer to measure the physical activity and underwent a bioelectrical impedance analysis to determine the physical characteristics. Macro-nutrient was also surveyed.

RESULTS: The mean total CTTs (TCTT) in the males and females were 8.8 and 24.7 h (*P* = 0.002), respectively. In the male subjects, the right CTT (3.5 ± 4.9 h *vs* 10.0 ± 11.6 h, *P* = 0.023) and recto-sigmoid CTT (4.4 ± 4.7 *vs* 13.6 ± 12.5 h, *P* = 0.004) were significantly shorter and the total energy expenditure (637.6 ± 44.3 kcal *vs* 464.3 ± 64.9 kcal, *P* = 0.003), total activity count (247 017 ± 75 022 count *vs* 178 014 ± 75 998 count, *P* = 0.003), energy expenditure of light intensity (148.5 ± 6.9 kcal *vs* 120.0 ± 16.8 kcal, *P* = 0.006), energy expenditure of moderate intensity (472.0 ± 36.2 kcal *vs* 281.4 ± 22.2 kcal, *P* < 0.001), fat intake (65.5 ± 23.3 g *vs* 51.2 ± 17.4 g, *P* = 0.010), and water consumption (1714.3 ± 329.4 g *vs* 1164.7 ± 263.6 g, *P* = 0.009) were significantly higher than in the female subjects. Regarding correlations, when adjusted for gender, fiber (*r* = -0.545, *P* < 0.001) and water intake (*r* = -0.257, *P* < 0.05) correlated significantly with the TCTT in all subjects. In addition, the body mass index (*r* = -0.424, *P* < 0.05) and fiber intake (*r* = -0.417, *P* < 0.05) in the males as well as the fiber intake (*r* = -0.655, *P* < 0.001) in the females showed significant correlations with the TCTT.

CONCLUSION: The subjects showed significant gender differences in the TCTT, right CTT, and recto-sigmoid CTT. Furthermore, the intake of the fiber and water contributed to the CTT.

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Key words: Colon transit time; Physical activity; Characteristics; Macro-nutrient; South Korean

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INTRODUCTION

Colorectal cancer is the third most prevalent human cancer worldwide, with 1 million estimated new case annually, of which, about 50% lead to death[1]. The recog-

nized risk factors include westernized eating habits, obesity, insufficient physical activity and genetic factors[2,3]. However, the effects of gender and individual behavior on the colon transit time (CTT) remain controversial. In some studies, some factors affecting the CTT include age, gender, body mass index (BMI), dietary fiber, water intake, and living habits[4-6]. On the other hand, in a literature review of factors affecting the CTT, age[7], gender[2,3,7], BMI[8], and dietary fiber[9] were not found to be statistically significant.

The impact of exercise and physical activity (PA) on the gastrointestinal and colonic tracts is an area of emerging interest, with recent research focusing on the potential benefits of PA[3,4]. PA is known to be associated with colonic function and constipation[9,10]. In addition, walking, running, and strength training are known to reduce the CTT[10,11]. Also, a period of physical inactivity in subjects who had been engaged in regular PA for several years significantly increased the CTT[12,13]. At present, leisure time activities and transport activities constitute the majority of one's daily physical over all activity[14-16]. Increasing PA is an important health goal for everyone, but doing this requires an accurate method of measuring daily PA. To solve this problem, accelerometers are now being utilized in studies of PA. Accelerometers measure the acceleration of movement and can quantify the intensity, frequency and duration of movement. Lightweight, small and very durable, accelerometers are practical for all ages and activities and can be used indoors or outdoors. Several studies to date have supported the use of accelerometers to assess energy expenditure during locomotion and have confirmed a validity range of 0.77-0.89[11,12,16,17]. For this reason, to measure PA, we analyzed total energy expenditure and energy expenditure of low-, moderate-, and vigorous intensity physical activities using an accelerometer.

Measuring the CTT by the radio-opaque marker method is simple, widely available and important for the diagnosis of slow transit constipation[18-20]. Although there are several reports which sought to measure the CTT using Kolomark™, a radio-opaque marker, normative data on South Koreans are still lacking. Thus, to understand colonic motor function in Koran adults, the CTT was analyzed using Kolomark™ in the present study. To investigate the factors which influence the CTT in South Korean adults, we examined the effects of gender, physical characteristics, physical activity, and macro-nutrient and water intake on the CTT.

MATERIALS AND METHODS

Participants

The study subjects were 47 adults, 23 of whom were male and 24 female. The mean age was 36.0 years with a range of 25-59 years, and all voluntarily gave written informed consent. Of all potential subjects, those with any possible restriction on their normal PA or diet, those with cardiovascular or orthopedic disease which may affect the CTT, menstruating woman those who cannot ingest drugs due to functional stomach diseases, and those on any prescription of anti-constipation drugs or for diabetes mellitus or hypertension were excluded from the current analysis.

Measurement of physical characteristics

A bioelectrical impedance analysis (Inbody, Biospace, Seoul, South Korea) was used to measure the height, weight, BMI, lean body mass (LBM), and percentage of body fat (BF) at the beginning of the study in a fasted state.

Measurement of physical activity

PA was measured over a 1-wk period using an accelerometer (Actical, Mini Mitter, Chicago, IL, United States). To measure PA accurately, the study subjects were advised to perform their daily PA as they normally would. The accelerometer was attached to the iliac crest using a belt. Prior to measurement, the age, sex, height and weight of each participant were entered into the device. The total energy expenditure, activity energy expenditure, and time of activity depending on the PA intensity and frequency were individually measured according to the time. The results were automatically stored. Using the recorded data, the energy expenditure was calculated based on Muffin's formula to calculate the basal metabolic rate[11,17].

Analysis of macro-nutrient intake

To determine the average daily energy, carbohydrate, protein, fat, fiber and water intake, all participants recorded their daily dietary information using the dietary recall method over a 1 wk period during the accelerometer phase. Foods that could not fit into a standard measure were described by the dimensions of their shape, with a grid measured in centimeters to allow these measurements to be done accurately. The dietary records were analyzed using the nutrient analysis program.

Measurement of the CTT

To measure the colonic motor function, the CTT was measured using a multiple marker technique with a radio-opaque marker (Kolomark™, MI Tech, Pyeongtaek, South Korea). The subjects took one capsule containing 20 radio-opaque markers at the same time every day for three consecutive days. On the fourth day, following the first administration, a supine abdominal radiograph was performed. The mean CTT (hours) was calculated by counting the number of radio-opaque markers that were left in the total colon and in each segment of the colon and then multiplying this number by 1.2[18-20].

Ethics

This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Institutional Review Board of Seoul National University (IRB No. 2011/1006). All patients provided informed written consent.

Table 1 Physical characteristic of the participants

Variable	Male (n = 23)	Female (n = 24)	Total (n = 47)	P value
Age (yr)	37.3 ± 5.8	35.9 ± 5.6	36.0 ± 5.9	0.108
Height (cm)	172.1 ± 5.1	159.6 ± 6.3	165.2 ± 8.5	< 0.001
Weight (kg)	75.6 ± 12.5	55.2 ± 6.8	64.4 ± 14.1	< 0.001
BMI (kg/m^2)	24.5 ± 3.5	21.7 ± 2.5	23.4 ± 3.5	< 0.001
LBM (kg)	57.1 ± 15.3	41.1 ± 5.0	48.3 ± 13.5	< 0.001
BF (%)	21.1 ± 8.7	25.5 ± 3.5	23.5 ± 6.7	0.009

Data are presented as mean ± SD. BMI: Body mass index; LBM: Lean body mass; BF: Body fat.

Table 2 Physical activity amount and intensity of the subjects

Variable	Male	Female	Total	P value
TEE (kcal)	637.6 ± 44.3	464.3 ± 64.9	542.4 ± 54.9	0.003
TAC (count)	247 017 ± 75 022	178 014 ± 75 998	212 515.5 ± 75 501	0.003
EEL (kcal)	148.5 ± 6.9	120.0 ± 16.8	133.1 ± 16.8	0.006
EEM (kcal)	472.0 ± 36.2	281.4 ± 22.2	367.4 ± 22.2	< 0.001
EEV (kcal)	24.5 ± 6.7	11.1 ± 47.8	17.8 ± 13.8	0.214

Data are presented as mean ± SD. TEE: Total energy expenditure; TAC: Total activity count; EEL: Energy expenditure of light intense activity; EEM: Energy expenditure of moderate intense activity; EEV: Energy expenditure of vigorous intense activity.

Table 3 Daily macro-nutrients intake of the subjects

Variable	Male	Female	Total	P value
Totalenergy (kcal)	2193.1 ± 259.2	2091.5 ± 229.2	2147.2 ± 233.6	0.292
Protein (g)	85.0 ± 18.6	82.5 ± 20.9	83.6 ± 19.6	0.493
Fat (g)	65.5 ± 23.3	51.2 ± 17.4	59.0 ± 21.9	0.010
Carbohydrate (g)	338.8 ± 71.7	298.5 ± 42.4	305.8 ± 61.2	0.054
Fiber(g)	8.1 ± 4.5	9.9 ± 4.7	8.9 ± 4.6	0.133
Water (g)	1714.3 ± 329.4	1164.7 ± 263.6	1227.0 ± 286.8	0.009

Data are presented as mean ± SD.

Table 4 Segmental colon transit time of the subjects

Variable	Male	Female	Total	P value
LCTT (h)	0.9 ± 2.2	1.1 ± 3.0	1.0 ± 2.6	0.592
RCTT (h)	3.5 ± 4.9	10.0 ± 11.6	6.8 ± 9.6	0.023
RSCTT (h)	4.4 ± 4.7	13.6 ± 12.5	9.0 ± 8.6	0.004
TCTT (h)	8.8 ± 9.4	24.7 ± 23.6	16.8 ± 16.2	0.002

Data are presented as mean ± SD. RCTT: Right colon transit time; LCTT: Left colon transit time; RSCTT: Recto-sigmoid colon transit time; TCTT: Total colon transit time.

Statistical analysis

Data analysis was conducted using the SPSS software package (version 18.0, SPSS Inc., Chicago, IL, United States), and descriptive statistics were presented using mean ± SD. The differences in the variables between the males and females were verified by an independent samples t-test. To assess the correlation between the total CTT and all of the experiment variables, Partial's correlation coefficient (r) was determined. An adjustment was made for gender. The level of significance (P) was 0.05 for all statistical analyses.

RESULTS

Physical characteristics

The physical characteristics of all subjects are presented in Table 1. The mean age was 37.3 and 35.9 years in the male and female subjects, respectively. The variables associated with the physical characteristics of the height, body weight, BMI and LBM were significantly higher in the males than in the females ($P < 0.001$). On the other hand, the BF was significantly lower in the male subjects than in the female subjects ($P = 0.009$).

Physical activity amount and intensity

The amounts and intensities of the PA of the male and female subjects are shown in Table 2. The total energy expenditure (173.3 kcal, $P = 0.003$), total activity count (69 003 count, $P = 0.003$), energy expenditure of light intensity (EEL) (28.5 kcal, $P = 0.006$), and energy expenditure of moderate intensity (EEM) (190.6 kcal, $P < 0.001$) were significantly higher in the male subjects than in the female subjects.

Macro-nutrient intake

Daily macro-nutrient and water intake by the subjects are presented in Table 3. The intake levels of fat (14.3 g, $P = 0.010$) and water (549.6 g, $P = 0.009$) were significantly higher in the males than in the females.

Segmental CTT and total CTT

The mean total CTT (TCTT) was 8.8 h and 24.7 h in the male and female subjects, respectively. This gender difference was statistically significant ($P = 0.002$). Although there was no significant difference in the left CTT, the right CTT ($P = 0.023$) and recto-sigmoid CTT ($P = 0.004$) showed significant gender difference (Table 4).

Correlation between the TCTT and parameters

Correlations between the TCTT and related parameters for the male and female subjects are shown in Table 5. In the male subjects, the BMI ($r = -424$, $P < 0.05$) and fiber intake levels ($r = -417$, $P < 0.05$) showed significant correlations with the TCTT. On the other hand, only the fiber intake ($r = -655$, $P < 0.001$) showed a significant correlation with the TCTT in the female subjects. Moreover, when adjusted for gender, the fiber ($r = -545$, $P < 0.001$) and water ($r = -257$, $P < 0.05$) intake levels showed significant correlations with the TCTT in all subjects.

DISCUSSION

All physical characteristics were higher in the male subjects, except for the BF, but even those values were within the normal range. These values indicate that the South Korean adults who participated in this study were relatively healthy.

Table 5 Correlations between the total colon transit time and parameters

Variable	Male	Female	Total[1]
Physicalcharacteristics			
Age	0.060	-0.112	-0.067
Height	0.071	-0.157	-0.108
Weight	-0.344	-0.014	-0.098
BMI	-0.424	0.127	-0.031
LBM	-0.053	-0.107	-0.050
BF	-0.243	0.149	0.051
Physicalactivity			
TEE	-0.244	-0.022	-0.031
TAC	-0.191	-0.164	-0.180
EEL	-0.067	-0.211	-0.182
EEM	-0.197	-0.092	-0.101
EEV	-0.256	0.092	0.078
Macro-nutrientintake			
Energy	0.196	0.158	0.290
Protein	-0.102	-0.247	-0.123
Fat	0.024	-0.155	-0.008
Carbohydrate	0.346	-0.221	0.250
Fiber	-0.417	-0.655	-0.545
Water	-0.266	-0.326	-0.257

[1]Adjusted for gender. BMI: Body mass index; LBM: Lean body mass; BF: Body fat; TEE: Total energy expenditure; TAC: Total activity count; EEL: Energy expenditure of light intense activity; EEM: Energy expenditure of moderate intense activity; EEV: Energy expenditure of vigorous intense activity.

Despite longstanding disputes, it is uncertain as to whether or not there is any difference in the CTT between males and females. In our study, the comparison of the TCTTs according to gender showed that the mean TCTTs of the males and females were 8.8 and 24.7 h, respectively. Drossman *et al*[21] and Heaton *et al*[22] reported that the prevalence of constipation is higher in women in than in man, and that it becomes more prominent in woman of childbearing age, suggesting a role of the female sex hormone. Jung *et al*[6] reported that the mean TCTT of healthy South Korean subjects was 22.3 h in male subjects and 30.1 h in female subjects and that was no significant difference in the TCTT between male and female subjects. In our study, however, a significant difference was noted in the TCTT according to gender. Moreover, the RCTT and RSCTT in females showed were significantly longer than they were in males. A radio-opaque marker study can provide us with information about the segmental CTT. Our study showed that gender and the LCTT were unrelated. Jung *et al*[6] showed that the RSCTT was significant longer in the female subjects, whereas TCTT, RCTT, and LCTT differences in terms of gender were not significant. Rao *et al*[23] in a study that used ambulatory 24 h colonic manometry revealed that women showed less pressure activity than men and that this difference was particularly significant in the transverse descending colon. Therefore, a gender difference may contribute to the prevalence of constipation in women. However, more studies of variables and sample sizes regarding this mechanism are required.

Because a sedentary lifestyle is a risk factor for chronic disease, promoting PA is very important[24,25]. Some studies reported that factors that can influence the CTT are regular PA and regular physical exercise, both of which are considered to be useful in the management of chronic constipation[26,27]. This is based partly on the assumption that exercise shortens the CTT through the gastrointestinal tract. However, studies testing the influence of exercise on the CTT in healthy young subjects show conflicting results. Some studies showed a decrease in the CTT after physical training[11-13], whereas other studies did not show a reduction partly owing to large intra-individual differences[28-30]. In our study, although South Korean male adults engaged in more PA with or without an adjustment for gender, the CTT did not correlate with the PA levels.

Regarding the intake of dietary fiber and water, several studies have shown that the CTT was shortened and the stool frequency increased when the amounts of dietary fiber and water increased[31-34]. In our study, the mean CTT also showed a significant correlation with the amount of dietary fiber intake in all subjects. This result is similar to those of previous studies, which found that the CTT is significantly correlated with dietary fiber intake. We observed an inverse association between the TCTT and fiber intake in males, females, and in all subjects with or without an adjustment for gender. This result indicates that fiber intake is a strong contributing factor which affects the CTT in South Korean adults. For water intake, although all subjects showed significant correlation between this and the TCTT, the discrepancy in water consumption may be due to the limitation of the measurement methods based on the subjects' food diaries. The Can-pro program by the Korean Nutrition Society does not analyze the moisture content of food. Thus, the surveyed amounts of consumed water were used for the water intake amounts. As a result, the amounts of water contained in the subject's food intake level were excluded from total water consumption in our study. However, although our ability to analyze water consumption was limited, water consumption was shown to help the CTT in all subjects in the present study. The current study was also limited by the small sample size, which prevents any generalization of the study results. Therefore, further studies should be conducted with more subjects.

Other factors that can influence the CTT include physical characteristics such as weight, and height and age[4,5]. In our study, the average BMI of the subjects was 24.5 kg/m^2 for the men and 21.7 kg/m^2 for the women. Considering that the normal range of BMI reported by the World Health Organization was 18.5-25.0 kg/m^2 for adults, these values indicate that the South Korean adults who participated in this study were relatively healthy. The finding of a correlation between the TCTT and BMI in the male subjects, but not in the female subjects, showed that the BMI increased the CTT, as in previous studies. The precise mechanism between the BMI and CTT could not be explained, although possible reasons include differences in eating habits and living patterns.

In our study, the BMI was a factor contributing to the CTT in only males. Martelli *et al*[4] showed that the CTT of healthy older subjects (age 55-74 years) was longer on average than that of healthy young subjects (age 21-27 years). However, in our study, age did not correlate with the CTT for those aged 25-59 years.

Based on these results, significant gender differences were noted in the TCTT, RCTT and RSCTT. The BMI and fiber intake in males and fiber intake in females were shown to assist the CTT. Moreover, the intake of the fiber and water intake was shown to help the CTT in all subjects.

COMMENTS

Background

The recognized risk factors of colorectal cancer include westernized eating habits, obesity, insufficient physical activity and genetic factors. However, the effects of gender and individual behavior on the colon transit time (CTT) remain controversial.

Research frontiers

The impact of exercise and physical activity (PA) on the gastrointestinal and colonic tracts is an area of emerging interest, with recent research focusing on the potential benefits of PA.

Innovations and breakthroughs

The authors measured physical activity by accelerometer, colon transit time by radio-opaque marker method and food intake by the nutrient analysis program that are all very good and relevant. Colon transit time in South Korean general population showed gender difference and modulation of CTT by the intake of fiber and water.

Applications

CTT has a gender difference which are involved total calories, energy expenditure, physical activity, and water consumption. Regardless the gender, the intake of fiber and water modulated to reduce colon transit time. It can help the general advise for life habit with patients of constipation. A further study that including larger sample size and physical activity intervention may assist to elucidate the precise relationship between physical activity and colon movement.

Peer review

The authors have studied CTT according to physical activity and characteristics in South Korean, finding that significant gender differences in the total CTT, right CTT, and recto-sigmoid CTT adults. The manuscript is a good study and should be acceptable.

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BRIEF ARTICLE

New endoscopic classification of achalasia for selection of candidates for peroral endoscopic myotomy

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Abstract

AIM: To propose a new endoscopic classification of achalasia for selecting patients appropriate for undergoing peroral endoscopic myotomy (POEM).

METHODS: We screened out the data of patients with achalasia examined from October 2000 to September 2011 at our Digestive Endoscopic Center with endoscopic pictures clear enough to reveal the morphology of middle and lower esophagus. After analyzing the correlation between the endoscopic morphology of the esophageal lumen and POEM, we proposed a new endoscopic classification (Ling classification) of achalasia according to three kinds of endoscopically viewed structures: multi-ring structure, crescent-like structure and diverticulum structure. There were three types based on the criteria of Ling classification: type Ⅰ, smooth without multi-ring, crescent-like structure or diverticulum structure; type Ⅱ, with multi-ring or crescent-like structure but without diverticulum structure; and type Ⅲ, with diverticulum structure. Type Ⅱ was classified into three subtypes: Ling Ⅱa, Ling Ⅱb and Ling Ⅱc; and type Ⅲ also had three subtypes: Ling Ⅲl, Ling Ⅲr and Ling Ⅲlr. Two endoscopists made a final decision upon mutual agreement through discussion if their separately recorded characteristics were different.

RESULTS: Among the 976 screened patients with achalasia, 636 patients with qualified endoscopic pictures were selected for the analysis, including 405 males and 231 females. The average age was 42.7 years, ranging from 6 to 93 years. Type Ⅰ was the most commonly observed type of achalasia, accounting for 64.5% (410/636), and type Ⅲ was the least commonly observed type of achalasia, accounting for 2.8% (18/636). And type Ⅱ accounted for 32.7% (208/636) and subtype of Ling Ⅱa, Ling Ⅱb and Ling Ⅱc accounted for 14.6% (93/636), 9.9% (63/636) and 8.2% (52/636), respectively. And subtype of Ling Ⅲl, Ling Ⅲr and Ling Ⅲlr accounted for 0.8% (5/636), 0.3% (2/636) and 1.7% (11/636), respectively.

CONCLUSION: A new endoscopic classification of achalasia is proposed that might help in determining the proper candidates for POEM.

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Key words: Endoscopy; Classification; Achalasia; Selection; Peroral endoscopic myotomy

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INTRODUCTION

Treatments for achalasia include pharmacological therapy, endoscopic injection of botulinum toxin, endoscopic dilation, and laparoscopic or open surgery[1-5], among which laparoscopic surgery with fundoplication has been widely accepted as the primary therapy because of its low complication rate, durable symptom relief, and low incidence of postoperative gastroesophageal reflux[6-10]. However, a new alternative therapy named peroral endoscopic myot-

omy (POEM) has recently been reported to be effective clinically for esophageal achalasia without serious complications at least in a short term[11-13]. We have performed POEM since 2010, and found that not all the patients with achalasia were eligible to undergo POEM. If the esophageal lumen was too tortuous or if a diverticulum was formed in the middle or the lower part of the esophagus, to establish an appropriate submucosal tunnel would, to a great extent, cause a penetrative damage to the mucosa or the muscularis propria, which obviously destroyed the completeness of the submucosal tunnel, leading to a serious outcome such as mediastinitis if not successfully treated in time. Mucosal penetration at the gastric cardia has been firstly reported to occur respectively in 2 out of 5 and 2 out of 17 patients in two studies, although it did not cause serious consequences[12-14]. The occurrence of mucosal penetration at the cardia was also confirmed by several other studies[15-19]. Therefore, it is very important and necessary to make a precise judgment whether or not a patient with achalasia is a proper candidate for POEM. Inoune *et al*[12] suggested that a patient was not fit for a POEM therapy if a double-lumen sign was detected in the esophageal computed tomography (CT). However, no other studies have, to our best knowledge, reported the correlation between the morphology of the esophageal lumen and the indications of POEM. To better judge if a patient was suitable for POEM, we analyzed the correlation between the endoscopic morphology of the esophageal lumen and POEM, and proposed a new endoscopic classification of achalasia (Ling classification), hoping to assist in determining the indications of POEM.

MATERIALS AND METHODS

We reviewed the data of endoscopic examinations of patients diagnosed with achalasia from October 2000 to September 2011 at our Digestive Endoscopic Center and screened out the data of patients with endoscopic pictures clear enough to reveal the morphology of the middle and the lower parts of the esophagus. Two endoscopists (Li HK and Linghu EQ) independently recorded the characteristics of the morphology of the middle and the lower parts of the esophagus, which included three endoscopically detected intraluminal structures: multi-ring structure, crescent-like structure and diverticulum structure. There were three types according to the Ling classification[20]: type Ⅰ, smooth without multi-ring, crescent-like structure or diverticulum structure; type Ⅱ, with multi-ring or crescent-like structure but without diverticulum structure; and type Ⅲ, with diverticulum structure (Figure 1). Type Ⅱ was further divided into three subtypes: Ling Ⅱa, Ling Ⅱb and Ling Ⅱc, and type Ⅲ also had three subtypes: Ling Ⅲl, Ling Ⅲr and Ling Ⅲlr. The criteria for classifying subtypes Ⅱ and III were as follows: Ling Ⅱa, with multi-ring structure; Ling Ⅱb, with crescent-like structure and the midpoint of its inner edge not larger than 1/3 of the esophageal lumen; Ling Ⅱc, with crescent-like structure and the midpoint of its inner edge over 1/3 of the esophageal lumen; Ling Ⅲl, diverticulum structure in the left wall of esophagus; Ling Ⅲr, diverticulum structure in the right wall of esophagus; and Ling Ⅲlr, diverticulum structure in both the left and right walls of esophagus. If the two endoscopists recorded different characteristics, they would discuss together and made a final decision upon mutual agreement.

RESULTS

Data of 976 patients with achalasia were collected and analyzed, and 636 of them were screened out with clear endoscopic pictures revealing the morphology of middle and lower esophagus, while 340 were excluded for unqualified pictures. Among the 636 patients, 405 were male and 231 were female. The average age was 42.7 years, ranging from 6 to 93 years.

We named our proposed classification Ling classification since Ling is short for Linghu, which is the family name of the corresponding author. As shown in Table 1, 64.5% (410/636) of the patients were classified as type Ⅰ, which was the most common type of achalasia based on the Ling classification; 32.7% (208/636) of the patients were classified as type Ⅱ; and only 2.8% (18/636) as type Ⅲ. The typical pictures of each type or subtype are shown in Figure 1. The proportion of each type or subtype is described in Table 1 according to the Ling classification.

DISCUSSION

As early as in 1980, Ortega *et al*[21] for the first time performed cutting esophageal muscles using the needle knife through the peroral endoscope, but few doctors followed them due to the possible mediastinal contamination. Until 2007, Pasricha *et al*[22] reported the possibility of POEM through a submucosal tunnel in a porcine model. In 2010, Inoue *et al*[12] started to use POEM to treat patients with achalasia after several refinements, including applications of endoscopic submucosal dissection technique, the triangle-tip knife, positive pressure ventilation and CO_2 insufflations through the endoscope during POEM, and they reported a satisfactory short-term effects. Swanström *et al*[13] also reported their experience of POEM in patients with achalasia and esophageal motility disorders. Both of the above-mentioned clinical trials demonstrated that small mucosal penetration at the gastric cardia occurred respectively in 2 out of 5 and 2 out of 17 patients, but were treated differently. Inoue observed the penetrations without special treatment, while Swanström used traditional hemostatic clips to seal the penetrations. Even though no serious clinical outcomes were observed, we should keep alert about the incompleteness of the submucosal tunnel caused by mucosal penetration at the cardia, and we used fibrin sealant to treat this mucosal penetration[15]. If the penetrations were *in situ*, fluids from the stomach or the esophagus might flow into the tunnel, which might affect the healing of the incised inner circular muscles and further influence the effects of POEM.

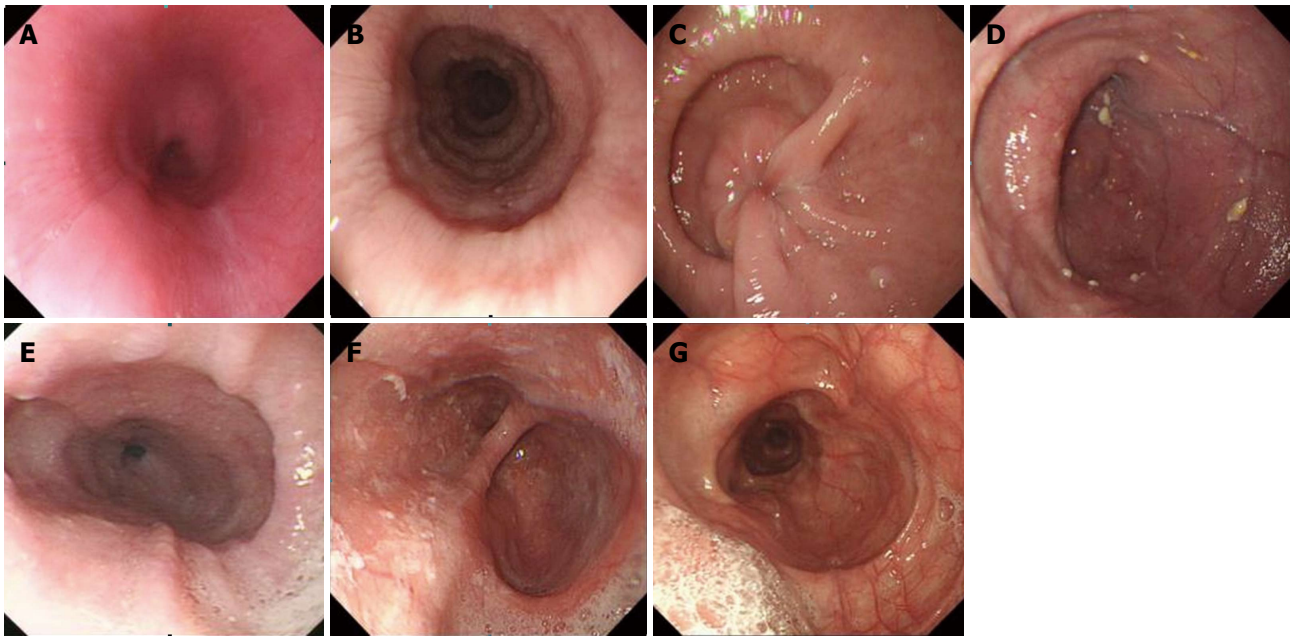

Figure 1 Typical pictures of each type or subtype in Ling classification. A: Type Ⅰ; B: Type Ⅱa; C: Type Ⅱb; D: Type Ⅱc; E: Type Ⅲl; F: Type Ⅲr; G: Type Ⅲlr.

Table 1 Proportion of each type or subtype according to Ling classification *n* (%)

Type	Proportion
Ling Ⅰ	410 (64.5)
Ling Ⅱ	208 (32.7)
Ling Ⅱa	93 (14.6)
Ling Ⅱb	63 (9.9)
Ling Ⅱc	52 (8.2)
Ling Ⅲ	18 (2.8)
Ling Ⅲl	5 (0.8)
Ling Ⅲr	2 (0.3)
Ling Ⅲlr	11 (1.7)

We think that the reason why mucosal penetrations are more likely to occur in the cardia is that the working space in the submucosal tunnel near the cardia is narrower than in other parts, which makes a precise electrocautery without damaging the cardiac mucosa very difficult. Similarly, if POEM was performed in patients with a very tortuous esophagus, penetrations of mucosa or muscularis propria could happen where the working space is confined by the tortuousness of the esophagus. So, from the point of minimizing complications like penetrations of mucosa or muscularis propria, it is very important to make a precise judgment whether a patient with achalasia is or not a proper candidate for POEM. Inoune mentioned that a patient was not fit for a POEM therapy if a double-lumen sign was detected in the CT scan[12], however, no other studies on POEM have reported the correlation between the morphology of esophageal lumen and the indications of POEM[13-14,16-18]. We, therefore, proposed the endoscopic Ling classification of achalasia, hoping to assist in determining the indications of POEM after analyzing the correlation between the endoscopic morphology of the esophageal lumen and POEM.

After reviewing the endoscopic pictures of nearly 1000 patients, we screened out 636 patients with pictures clear enough to reveal the middle and lower esophagus and analyzed the characteristics of the morphology of esophagus. Because the submucosal tunnel established during POEM generally starts from the middle esophagus and ends at about 3 cm distal to the cardia[12,13], we focused on the morphology of the middle and lower esophagus. Based on the Ling classification, the esophagus of type Ⅰ is very smooth and without multi-ring structure, crescent-like structure or diverticulum structure, so type Ⅰ patients are the most safe type to undergo POEM. The esophagus of type Ling Ⅱa with multi-ring structure was not so smooth as type Ⅰ, but not much difficulties were encountered during establishment of the submucosal tunnel in our practice of POEM. The crescent-like structure present in type Ling Ⅱb or Ling Ⅱc actually reflexes the tortuousness of the esophageal lumen, and the degree of tortuousness is more severe in Ling Ⅱc than in Ling Ⅱb, and therefore to establish a submucosal tunnel across the tortuous point of the esophagus is more difficult and more likely to cause a penetration in Ling Ⅱc than in Ling Ⅱb. Diverticulum structure occurred in type Ⅲ, making it the most challenging type to establish a submucosal tunnel across the diverticulum.

Location of the crescent-like structure or the diverticulum structure can greatly affect the success of a POEM therapy. Since we and other operators[12,13] preferred the establishment of submucosal tunnel in the right wall of the esophagus leading to the lesser curvature of stomach, the submucosal tunnel would be very difficult to be established if a crescent-like structure or diverticulum structure was located in the right wall of the esophagus. Otherwise, if a crescent-like structure or diverticulum

structure was located in the left wall of the esophagus, the submucosal tunnel would be established with much less difficulty.

Based on our experiences, we suggest that type Ling I and Ling II$_a$ patients are safe for POEM, and that Ling II$_b$ patients can also be considered for POEM, but more cautions should be used to avoid damaging the mucosa or the muscularis propria when the submucosal tunnel is being established across the crescent-like structure. For Ling II$_c$ and Ling III patients, POEM can be performed when the crescent-like structure is on the left side of the esophageal lumen, but it is not recommended when the crescent-like structure is on the right side. These suggestions may be modified in the future with improvement of technology. For example, when a new and safe technique to establish a tunnel across a crescent-like structure is available, a POEM therapy would be recommended for all the Ling II patients free of concerns of penetrations irrespective of the location of the crescent-like structures.

One classification of achalasia categorized by high-resolution manometry has been reported to be promising in predicting the efficacy of treatment of achalasia, such as balloon dilation[23-25]. But up to now, there has been no report about its usefulness for selecting candidates for POEM. And the relationship between the proposed endoscopic classification and manometric classification remains an interesting point worth further studying.

Since not all patients were eligible for undergoing POEM, we analyzed the characteristics of the esophageal lumen and proposed the Ling classification, hoping that Ling classification might help in determining the proper candidates for POEM, thus minimizing the complications such as penetration of mucosa or muscularis propria because other therapies such as endoscopic injection of botulinum toxin, endoscopic dilation or surgery would be recommended instead of POEM in patients with a severely tortuous esophagus. However, further prospective studies are needed to determine the usefulness of Ling classification for selecting candidates for POEM.

COMMENTS

Background

Peroral endoscopic myotomy (POEM) has been primarily confirmed to be effective for patients with achalasia in the short term by several studies since it was first clinically reported in 2010, but not all the patients with achalasia were eligible for a POEM therapy. If the esophageal lumen was too tortuous or a diverticulum was formed in the middle or the lower part of the esophagus, it would be quite challenging to establish a submucosal tunnel.

Research frontiers

Although no serious clinical outcomes of complications like mucosal perforation in the cardia has been reported, it is still of vital importance to select proper candidates for POEM so as to minimize those complications.

Innovations and breakthroughs

The authors proposed a new endoscopic classification of achalasia after analyzing the endoscopic pictures which clearly revealed the morphology of middle and lower esophagus and this new Ling classification of achalasia might assist in determining the indications of POEM.

Applications

The authors suggest the followings for choosing the candidates for POEM: Ling I and Ling II$_a$ patients are safe for POEM, and Ling II$_b$ patients can also be considered for POEM but more cautions should be used to avoid damaging the mucosa or the muscularis propria when the submucosal tunnel is being established across the crescent-like structure. For Ling II$_c$ and Ling III patients, POEM can be performed when the crescent-like structure is on the left side of the esophageal lumen, but it is not recommended when the crescent-like structure is on the right side.

Terminology

POEM is a newly developed endoscopic therapy for achalasia by myotomy of the inner circular muscle of the lower esophagus, thus reducing the lower esophageal sphincter pressure and relieving symptoms of the patients.

Peer review

This study proposed a new endoscopic classification of achalasia based on the morphology of the middle and lower part of the esophagus, and role of the new classification in determining proper candidates for POEM was explored, and the authors suggested indications based on this new classification,which is very interesting. However, further prospective studies are needed to determine the validity of this Ling classification for selecting candidates for POEM.

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BRIEF ARTICLE

Pilot study on efficacy of reduced cathartic bowel preparation with polyethylene glycol and bisacodyl

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Abstract

AIM: To evaluate the efficacy of reduced cathartic bowel preparation with 2 L polyethylene glycol (PEG)-4000 electrolyte solution and 10 mg bisacodyl enteric-coated tablets for computed tomographic colonography (CTC).

METHODS: Sixty subjects who gave informed consent were randomly assigned to study group A, study group B or the control group. On the day prior to CTC, subjects in study group A were given 20 mL 40% wt/vol barium sulfate suspension before 3 mealtimes, 60 mL 60% diatrizoate meglumine diluted in 250 mL water after supper, and 10 mg bisacodyl enteric-coated tablets 1 h before oral administration of 2 L PEG-4000 electrolyte solution. Subjects in study group B were treated identically to those in study group A, with the exception of bisacodyl which was given 1 h after oral PEG-4000. Subjects in the control group were managed using the same strategy as the subjects in study group A, but without administration of bisacodyl. Residual stool and fluid scores, the attenuation value of residual fluid, and discomfort during bowel preparation in the three groups were analyzed statistically.

RESULTS: The mean scores for residual stool and fluid in study group A were lower than those in study group B, but the differences were not statistically significant. Subjects in study group A showed greater stool and fluid cleansing ability than the subjects in study group B. The mean scores for residual stool and fluid in study groups A and B were lower than those in the control group, and were significantly different. There was no significant difference in the mean attenuation value of residual fluid between study group A, study group B and the control group. The total discomfort index during bowel preparation was 46, 45 and 45 in the three groups, respectively, with no significant difference.

CONCLUSION: Administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution enhances stool and fluid cleansing ability, and has no impact on the attenuation value of residual fluid or the discomfort index. The former is an excellent alternative for CTC colorectum cleansing

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Key words: Colorectal cancer; Screening; Computed tomographic colonography; Polyethylene glycol; Bisacodyl

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INTRODUCTION

Computed tomographic colonography (CTC) has been shown to be an effective tool for colorectal cancer screening, due to its non-invasiveness and high sensitivity for polyp and neoplasia detection[1-6]. This sensitivity is comparable to that obtained using optical colonoscopy[7,8]. However, this examination still requires bowel cleansing similar to that used prior to optical colonoscopy[9]. The need for thorough colorectal cleansing using cathartics remains a major barrier limiting subject acceptance[10]. Reduced cathartic bowel preparation will increase subject compliance to CTC screening[11-13]. To date, there is no general consensus on reduced cathartic bowel preparation, which combines easy preparation and good acceptance[14-18].

Polyethylene glycol (PEG) electrolyte solution is an isosmotic laxative which does not cause electrolyte imbalance[19]. To date, a little research has been conducted on reduced cathartic bowel preparation with PEG-3350 and 10 mg bisacodyl for CTC; however, there are no studies on reduced cathartic bowel preparation combining PEG-4000 with 10 mg bisacodyl[9,12]. Hence, the purpose of this pilot study was to prospectively evaluate the efficacy of reduced cathartic bowel preparation with 2 L PEG-4000 electrolyte solution and 10 mg bisacodyl enteric-coated tablets for CTC.

MATERIALS AND METHODS

Study subjects

Our randomized, prospective and investigator-blinded study group was composed of 60 subjects (34 men and 26 women; age range 22-76 years, mean age 43.6 years). Indications for participation were as follows: asymptomatic subjects with increased colorectal cancer risk due to family or personal history; subjects with recent onset of alarm symptoms, i.e., positive fecal occult blood test, blood in feces, abdominal pain, alternating bowel, refractory iron-deficient anemia, constipation or diarrhea. Subjects with age younger than 18 years, inflammatory bowel disease, end-stage renal disease, and women of child-bearing age were excluded.

After a subject was enrolled in the study, he or she was assigned either to study group A, study group B or the control group on the basis of random numbers. The study protocol was approved by the institutional review board, and informed consent was obtained from each subject.

Bowel preparation

Subjects were instructed to avoid solid food on the day prior to the colorectal examinations. Subjects in study group A were given 20 mL 40% wt/vol barium sulfate suspension (Qingdao Dongfeng Chemical Co. Ltd., Shandong, China) before 3 mealtimes (breakfast, 7:00; lunch, 12:00; supper, 19:00) to achieve fecal tagging; for fluid tagging, 60 mL 60% diatrizoate meglumine (Hunan Hansen Pharmacy Co. Ltd, Hunan, China) diluted in 250 mL water was taken orally after supper; and then two 5 mg bisacodyl enteric-coated tablets (Shaanxi Chuanlong Pharmacy Co. Ltd, Shaanxi, China) were administered at 19:00; and finally, at 20:00, 2 L PEG-4000 electrolyte solution (Jiangxi Hengkang Pharmacy Co. Ltd, Jiangxi, China; each liter solution consisted of 15 mEq PEG-4000, 125 mEq Na^+, 10 mEq K^+, 20 mEq HCO_3^-, 80 mEq SO_4^{2-}, and 35 mEq Cl^-) was administered orally with the first dose of 750 mL, and the remainder was divided into five 250 mL aliquots separated by 15 min. Bowel preparation of the subjects in study group B was identical to that in study group A, with the exception of bisacodyl administration at 21:00. Bowel preparation in subjects in the control group was the same as that in study group A, without administration of bisacodyl.

CTC protocol

Anisodamine hydrochloride (10 mg) was injected intramuscularly 10 min before helical computed tomography (CT) scanning to allow optimal colonic distention, minimize peristalsis and alleviate spasms. Subjects were placed in the right lateral decubitus position on the CT table and a rectal catheter with a retention cuff was inserted. After inflation of the retention cuff, the cuff was gently pulled back until its proximal end rested on the anal sphincter. To distend the colorectum as fully as possible, subjects were then moved into the supine position and room air was gently insufflated into the colorectum to maximal subject tolerance using an automated delivery (JS-628, Guangzhou Jinjian Co. Ltd, Guangdong, China). The delivery was stopped if the rectal pressure was consistently over 3.5 kPa.

Scanning was performed with a 128-slice CT scanner (Somatom Definition AS 128, Siemens AG, Erlangen, Germany) or a 4-slice CT scanner (Aquilion 4, Toshiba Co., Japan) using the following parameters: collimation, 0.625; thickness, 1 mm; pitch, 1.2; electric current, 60 mA; voltage, 120 kV; matrix, 512 × 512; field of view, 3500-4000 mm. Subjects were instructed to hold their breath during data acquisition. A standard CT scout image of the abdomen and pelvis was acquired to assess the degree of colorectal distention, and more room air was insufflated if required. With the scout image, each examination was tailored to encompass the entire colorectum. Scanning was then performed in the prone position, using the same parameters as those in the supine position.

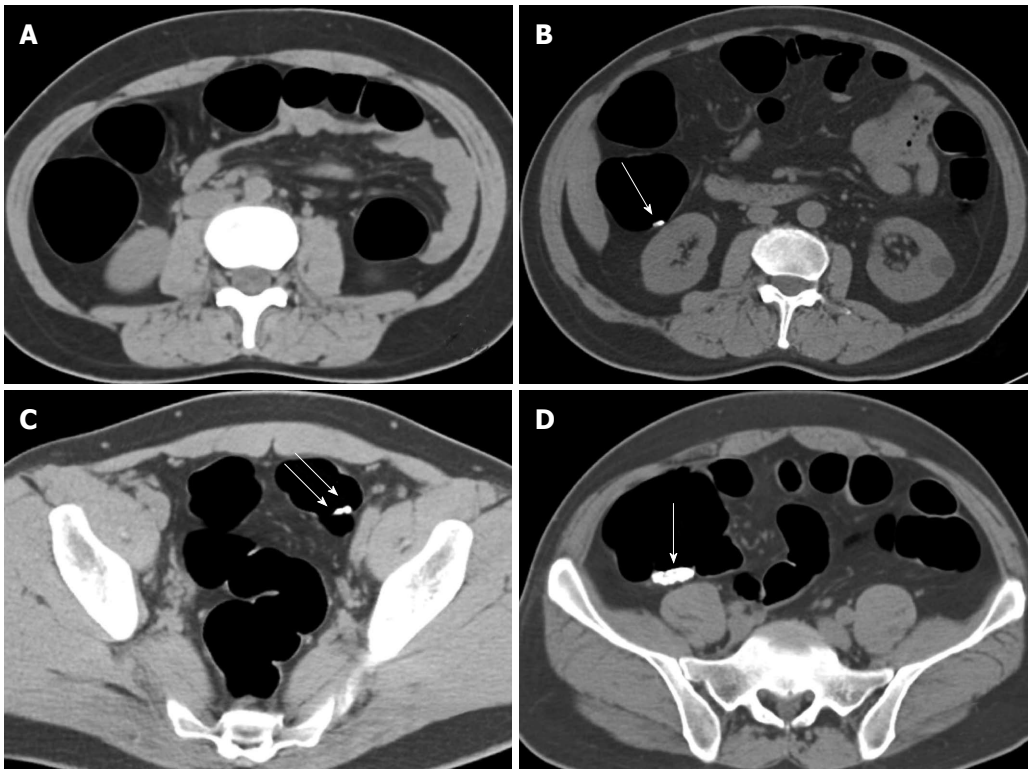

Figure 1 Four-point scoring system of residual stool. A: Stool score of 1: no residual stool; B: Stool score of 2: one stool particle (arrow) smaller than 5 mm in diameter; C: Stool score of 3: two stool particles (arrows) smaller than 5 mm in diameter; D: Stool score of 4: stool particle (arrow) larger than 5 mm in diameter.

Discomfort during bowel preparation

Subjects were invited to complete a questionnaire to describe their discomfort during bowel preparation. Possible discomfort included thirst, hunger, bloating, abdominal pain, sleep disturbance, malaise, nausea, vomiting, cramping, anal discomfort, dizziness and others.

Image evaluation

Axial two-dimensional images in the supine position were evaluated. Axial two-dimensional images in the prone position and multiplanar reformation images were also evaluated when necessary. All images were evaluated by two independent experienced readers, who were blind to histories, clinical symptoms and signs, and reduced cathartic regimens in the subjects, in random order on a picture archiving and communication system workstation with regard to residual stool, residual fluid and attenuation value of the residual fluid. If their evaluations differed, the two readers reached a consensus after reviewing and discussing the controversial images with another senior radiologist. The scores from the two readers were averaged to give an overall score for each segment in each subject. The entire colorectum was divided into six anatomic segments: cecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum.

Residual stool evaluation

A previously established four-point scoring system was used to evaluate residual stool in each of the six colorectum segments with lower scores corresponding to decreased residual stool[18]. A colorectum segment with no stool was given a score of 1; a segment with a single residual stool particle smaller than 5 mm in diameter, a score of 2; a segment with two or three particles of stool all smaller than 5 mm in diameter, a score of 3; and a segment with stool particles larger than 5 mm or more numerous than three, a score of 4 (Figure 1). A score of 1 or 2, indicated good stool cleansing; and a score of 3 or 4, indicated poor stool cleansing.

Residual fluid evaluation

A similar four-point scale was used to assess residual fluid with lower scores corresponding to a decreased percentage of the distended colorectum segment occupied by residual fluid[18]. The score reflected the percentage of the colorectum lumen filled with fluid. A segment with no fluid was given a score of 1; a segment with less than 25% of the lumen filled, a score of 2; a segment with 25%-50% of the lumen filled, a score of 3; and a segment with more than 50% of the lumen filled, a score of 4 (Figure 2). A score of 1 or 2, indicated good fluid cleansing; and a score of 3 or 4, indicated poor fluid cleansing.

Measurement of the attenuation value of residual fluid

The attenuation value of residual fluid was obtained by placing a single region of interest which was approximately 2 cm in diameter in the largest fluid collection. The mean of these measurements was calculated to establish the fluid attenuation value for each fluid collection.

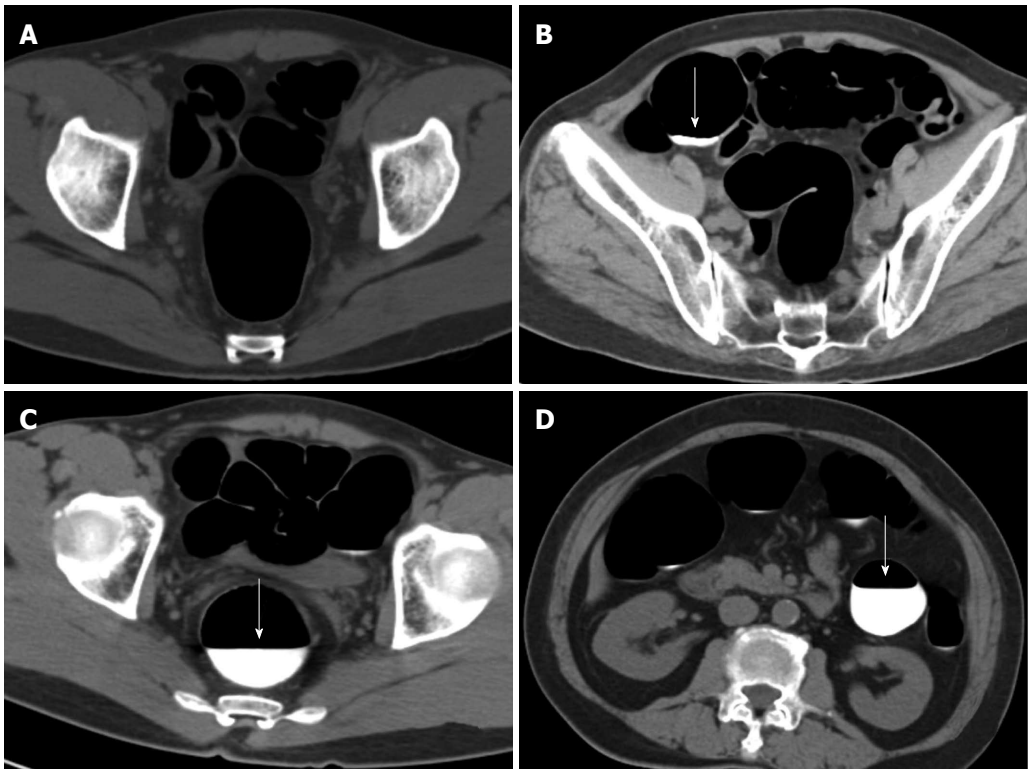

Figure 2 Four-point scoring system of residual fluid. A: Fluid score of 1: no residual fluid; B: Fluid score of 2: less than 25% of the lumen filled with fluid (arrow); C: Fluid score of 3: 25%-50% of the lumen filled with fluid (arrow); D: Fluid score of 4: more than 50% of the lumen filled with fluid (arrow).

Statistical analysis

Continuous data were expressed as means with standard deviations unless otherwise specified. Unpaired Student's *t* tests with the Welch correction were used to compare residual stool and fluid scores, attenuation value of residual fluid, and discomfort during bowel preparation among the three groups using SPSS version 13.0 for Windows. A P value < 0.05 was considered statistically significant.

RESULTS

Residual stool score

In study group A, a residual stool score of 1 was observed in 66 colorectum segments, 2 in 21 colorectum segments, 3 in 5 colorectum segments, 4 in 28 colorectum segments, with a mean score of 1.96 ± 0.11. In study group B, a residual stool score of 1 was observed in 68 colorectum segments, 2 in 15 colorectum segments, 3 in 5 colorectum segments, 4 in 32 colorectum segments, with a mean score of 2.01 ± 0.12. In the control group, a residual stool score of 1 was observed in 37 colorectum segments, 2 in 15 colorectum segments, 3 in 23 colorectum segments, 4 in 45 colorectum segments, with a mean score of 2.63 ± 0.12. There were no significant differences when study group A was compared with study group B ($P > 0.05$); however, when study group A was compared with the control group ($P < 0.001$), and study group B was compared with the control group ($P < 0.002$) significant differences were observed (Table 1).

Good and poor stool cleansing was 72.5% (87/120) and 27.5% (33/120), respectively, in study group A; 69.2% (83/120) and 30.8% (37/120), respectively, in study group B; and 43.3% (52/120) and 56.7% (68/120), respectively, in the control group.

Residual fluid score

In study group A, a residual fluid score of 1 was observed in 67 colorectum segments, 2 in 46 colorectum segments, 3 in 7 colorectum segments, 4 in 0 colorectum segments, with a mean score of 1.50 ± 0.06. In study group B, a residual fluid score of 1 was observed in 66 colorectum segments, 2 in 45 colorectum segments, 3 in 8 colorectum segments, 4 in 1 colorectum segment, with a mean score of 1.53 ± 0.06. In the control group, a residual fluid score of 1 was observed in 58 colorectum segments, 2 in 37 colorectum segments, 3 in 18 colorectum segments, 4 in 7 colorectum segments, with a mean score of 1.78 ± 0.08. There were no significant differences when study group A was compared with study group B ($P > 0.05$); however, when study group A was compared with the control group ($P < 0.05$), and study group B was compared with the control group ($P < 0.05$) significant differences were observed (Table 1).

Good and poor fluid cleansing was 94.2% (113/120) and 5.8% (7/120), respectively, in study group A; 92.5% (111/120) and 7.5% (9/120), respectively, in study group B; and 79.2% (95/120) and 20.8% (25/120), respectively, in the control group.

Attenuation value of residual fluid

In study group A, the attenuation value of residual fluid

Table 1 Residual stool and fluid score in the three groups *n* (%)

Group	Residual stool stool and fluid score				mean ± SD
	1	2	3	4	
Residual stool stool					
Study group A	66 (50.0)	21 (17.5)	5 (4.2)	28 (23.3)	1.96 ± 0.11
Study group B	68 (56.7)	15 (12.5)	5 (4.2)	32 (26.6)	2.01 ± 0.12
Control group	37 (30.8)	15 (12.5)	23 (19.2)	45 (37.5)	2.63 ± 0.12
Residual fluid score					
Study group A	67 (55.9)	46 (38.3)	7 (5.8)	0 (0.0)	1.50 ± 0.06
Study group B	66 (55.0)	45 (37.5)	8 (6.7)	1 (0.8)	1.53 ± 0.06
Control group	58 (48.4)	37 (30.8)	18 (15.0)	7 (5.8)	1.78 ± 0.08

in the cecum was 718 HU, ascending colon was 840 HU, transverse colon was 704 HU, descending colon was 761 HU, sigmoid colon was 694 HU and rectum was 655 HU, with a mean attenuation value of residual fluid of 729 HU. In study group B, the attenuation value of residual fluid in the cecum was 713 HU, ascending colon was 795 HU, transverse colon was 692 HU, descending colon was 665 HU, sigmoid colon was 532 HU, and rectum was 521 HU, with a mean attenuation value of residual fluid of 653 HU. In the control group, the attenuation value of residual fluid in the cecum was 647 HU, ascending colon was 662 HU, transverse colon was 593 HU, descending colon was 643 HU, sigmoid colon was 534 HU, and rectum was 503 HU, with a mean attenuation value of residual fluid of 597 HU. The *P* values in study group A, study group B and the control group were all > 0.05 and not statistically significant (Table 2).

Discomfort during bowel preparation

The three most common complaints related to bowel preparation were hunger (n = 43), bloating (n = 37), and thirst (n = 23). Other discomfort experienced during bowel preparation included nausea (n = 10), abdominal pain (n = 9), dizziness (n = 7), sleep disturbance (n = 6), and vomiting (n = 1). There were no serious adverse events during bowel preparation in the three groups. Total discomfort index during bowel preparation in study group A, study group B, and the control group were 46, 45 and 45, respectively, and the differences were not significant (P > 0.05, Table 3).

DISCUSSION

Despite being a preventable neoplasm, colorectal cancer is the fourth leading cause of cancer death in China[20]. Early detection and removal of the precursor lesion significantly reduces the incidence and mortality associated with this neoplasm[21,22]. CTC has been demonstrated to be a feasible and promising new technique in colorectal cancer screening[23-25]. However, with the current methods of CTC, it is necessary to undertake adequate colorectal cleansing to achieve acceptable CTC sensitivity and specificity, as excess stool creates pseudopolyps and obscures true soft-tissue polyp visualization[26,27]. Full bowel preparation with cathartics is the major barrier to screening, due to the discomfort and inconvenience of

Table 2 Attenuation value of residual fluid in the three groups

Group	Cecum	Asc c	Tra c	Des c	Sig c	Rectum	Mean
Study group A	718	840	704	761	694	655	729
Study group B	713	795	692	665	532	521	653
Control group	647	662	593	643	534	503	597

Asc c: Ascending colon; Tra c: Transverse colon; Des c: Descending colon; Sig c: Sigmoid colon.

the associated diarrhea, and negatively affects examination compliance[28]. Reduced cathartic bowel preparation will increase subject compliance, and is under extensive study at present[29-32]. There is no general consensus as to which reduced cathartic bowel preparation to use[33,34]. However, the prospect of replacing conventional preparation with reduced cathartics has driven researchers to identify a new method which combines diagnostic reliability, ease of preparation and subject acceptance[35].

PEG electrolyte solution is an isosmotic laxative which does not cause electrolyte imbalance, and results in improved tagging of residual stool. However, the use of PEG preparations often results in excess residual fluid within the colorectum[19]. As a stimulant laxative, bisacodyl decreases residual fluid. Hence, bowel preparation combining PEG electrolyte solution with bisacodyl enteric-coated tablet may have an ameliorative effect on colorectal cleansing. The standard volume of PEG-3350 is 4 L; each liter of PEG-3350 solution consists of 100 g PEG-3350, 7.5 g sodium sulfate, 2.7 g sodium chloride, 1.0 g potassium chloride, 5.9 g sodium ascorbate, and 4.7 g ascorbic acid. The common dosage of bisacodyl is 20 mg or 10 mg. In our study, a reduced volume of 2 L PEG-4000 electrolyte solution and 10 mg bisacodyl enteric-coated tablets were administered orally; each liter of PEG-4000 electrolyte solution consisted of 15 mEq PEG-4000, 125 mEq Na^+, 10 mEq K^+, 20 mEq HCO_3^-, 80 mEq SO_4^{2-}, and 35 mEq Cl^-. The molecular weight of PEG-4000 is greater than that of PEG-3350 and has higher viscosity and greater ability to form solids than PEG-3350, and their chemical performance is different.

In our series, the residual stool score in study group A (1.96 ± 0.11) was lower than that in study group B (2.01 ± 0.12) and the difference was not significant, which suggests that the cleansing stool ability was not affected by administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution. The residual stool score in study group A (1.96 ± 0.11) was lower than that in the control group (2.63 ± 0.12) and the difference was significant, which suggests that the stool cleansing ability was enhanced by administration of 10 mg bisacodyl enteric-coated tablets prior to oral administration of 2 L PEG-4000 electrolyte solution. The residual stool score in study group B (2.01 ± 0.12) was lower than that in the control group (2.63 ± 0.12) and the difference was significant, which suggests that oral administration of 10 mg bisacodyl enteric-coated tablets after oral administration of 2 L PEG-4000 electrolyte solution increases

Table 3 Discomfort during bowel preparation

Group	Hunger	Bloating	Thirst	Nausea	Abd p	Diz	Sle d	Vom	Total
Study group A	15	12	7	3	3	3	2	1	46
Study group B	14	13	8	3	3	2	2	0	45
Control group	14	12	8	4	3	2	2	0	45

Abd p: Abdominal pain; Diz: Dizziness; Sle d: Sleep disturbance; Vom: Vomiting.

stool cleansing ability. Therefore, oral administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution improves stool cleansing ability.

Although the residual fluid score in study group A (1.50 ± 0.06) was lower than that in study group B (1.53 ± 0.06) and the difference was not significant, which suggests that the fluid cleansing ability was not influenced by oral administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution. The residual stool score in study group A (1.50 ± 0.06) was lower than that in the control group (1.78 ± 0.08) and the difference was significant, which suggests that oral administration of 10 mg bisacodyl enteric-coated tablets prior to oral administration of 2 L PEG-4000 electrolyte solution heightened the fluid cleansing ability. The residual fluid score in study group B (1.53 ± 0.06) was lower than that in the control group (1.78 ± 0.08) and the difference was significant, which suggests that the fluid cleansing ability was improved by oral administration of 10 mg bisacodyl enteric-coated tablets after oral administration of 2 L PEG-4000 electrolyte solution. Hence, oral administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution increases the fluid cleansing ability. Borden *et al*[18] established a four-point scoring system to evaluate residual fluid in each of the six colorectum segments when comparing magnesium citrate and sodium phosphate. Using the same four-point scoring system, good and poor fluid cleansing was 52% and 48%, respectively, in the study by Hara *et al*[9] following the ingestion of 10 mg bisacodyl tablets after administration of 4 L PEG-3350. Good and poor fluid cleansing was 92.2% and 7.8%, respectively, in our study after oral administration of 10 mg bisacodyl enteric-coated tablets 1 h after oral administration of 2 L PEG-4000 electrolyte solution.

The mean attenuation value of residual fluid in study group A, study group B, and the control group was 729 HU, 653 HU and 597 HU, respectively, with no significant differences. This suggested that oral administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution did not affect the mean attenuation value of residual fluid. The attenuation value of residual fluid has been shown to have a significant effect on polyp conspicuity. Previous research has suggested that optimal viewing conditions in the two-dimensional format are met with an attenuation value of residual fluid of approximately 700 HU, due to high conspicuity of all polyps at this level. Such an increase in polyp conspicuity could lead to increased sensitivity and specificity of CTC. With a mean attenuation value of residual fluid of 729 HU, study group A was closer to this optimal level than study group B and the control group.

Total discomfort index during bowel preparation in study group A, study group B, and the control group was 46, 45 and 45, respectively; however, these differences were not significant. There were no serious adverse events during bowel preparation in the three groups. These results suggest that oral administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution does not influence subject discomfort during bowel preparation for CTC.

No significant differences were observed between study group A and B with regard to residual stool and fluid scores. However, the residual stool score in study group A (1.96 ± 0.11) was lower than that in study group B (2.01 ± 0.12), and the residual fluid score in study group A (1.50 ± 0.06) was also lower than that in study group B (1.53 ± 0.06), which indicated that oral administration of 10 mg bisacodyl enteric-coated tablets prior to oral administration of 2 L PEG-4000 electrolyte solution resulted in a trend toward greater stool and fluid cleansing ability than when given after PEG-4000 electrolyte solution. It would be preferable to administer 10 mg bisacodyl enteric-coated tablets prior to oral administration of 2 L PEG-4000 electrolyte solution for CTC bowel preparation.

There were two limitations in our pilot study. First, the number of subjects was relatively small and the study was likely underpowered to detect subtle differences in preparation adequacy. Second, we compared bowel preparation outcomes obtained with these three bowel preparation regimens, but did not assess the possible diagnostic performance of these regimens. Our study was not designed to address diagnostic performance, which will be studied at a later date.

In conclusion, oral administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution not only enhances the stool and fluid cleansing ability, but also has no impact on the attenuation value of residual fluid or discomfort during bowel preparation. Oral administration of 10 mg bisacodyl enteric-coated tablets prior to oral administration of 2 L PEG-4000 electrolyte solution is an excellent alternative for CTC colorectum cleansing.

COMMENTS

Background

Computed tomographic colonography (CTC) has been shown to be an effective tool for colorectal cancer screening. At present, this examination still requires bowel cleansing similar to that used prior to optical colonoscopy. The need for thorough colorectal cleansing using cathartics remains a major barrier limiting subject acceptance. Reduced cathartic bowel preparation will increase subject compliance in CTC screening. There is no general consensus on reduced cathartic bowel preparation.

Research frontiers

Reduced cathartic bowel preparation is under extensive study at present. The prospect of replacing conventional preparation with reduced cathartics has driven researchers to identify a new method which combines ease of preparation and subject acceptance.

Innovations and breakthroughs

Polyethylene glycol (PEG)-4000 electrolyte solution is an isosmotic laxative that does not cause electrolyte imbalance, and results in improved tagging of residual stool and excess residual fluid. Bisacodyl enteric-coated tablet, a stimulant laxative, decreases the residual fluid. Bowel preparation with combined PEG-4000 electrolyte solution and bisacodyl enteric-coated tablets has an ameliorative effect on colorectum cleansing.

Applications

Oral administration of 10 mg bisacodyl enteric-coated tablets prior to or after oral administration of 2 L PEG-4000 electrolyte solution enhances stool and fluid cleansing ability, and has no impact on the attenuation value of residual fluid or discomfort during bowel preparation. Oral administration of 10 mg bisacodyl enteric-coated tablets prior to oral administration of 2 L PEG-4000 electrolyte solution is an excellent alternative for CTC colorectum cleansing.

Terminology

CTC uses 2D computed tomographic (CT) images of the colorectum, rendered into 3D images and is used to screen for polyps and other abnormalities. The examination consists of non-invasive CT scans, obtained in a few minutes. At the workstation, the images are reconstructed into a 3D model of the colorectum, and the physician may begin clinical analysis of the images.

Peer review

It is a good reason for physician to find a convenient and effective method for patients to carry bowel preparation. But the cost and benefit and the discomfort score of the patient need to be considered.

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BRIEF ARTICLE

Retrospective study of steroid therapy for patients with autoimmune pancreatitis in a Chinese population

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Abstract

AIM: To explore the optimal steroid therapeutic strategy for autoimmune pancreatitis (AIP).

METHODS: This study was conducted retrospectively in two large institutions in China. Patients with clinically, radiologically and biochemically diagnosed AIP were enrolled. The performed radiological investigations and biochemical tests, the regimen of the given steroid treatment, remission and relapse whether with and without steroid therapy were analyzed.

RESULTS: Twenty-eight patients with AIP received steroid treatment, while 40 patients were treated surgically by pancreatoduodenectomy, distal pancreatectomy and choledochojejunostomy, radiofrequency ablation for the enlarged pancreatic head, percutaneous transhepatic biliary drainage and endoscopic biliary drainage. The starting oral prednisolone dose was 30 mg/d in 18 (64.3%) patients and 40 mg/d in 10 (35.7%) patients administered for 3 wk. The remission rate of AIP patients with steroid treatment (96.4%) was significantly higher than in those without steroid treatment (75%). Maintenance therapy (oral prednisolone dose 5 mg/d) was performed after remission for at least 6-12 mo to complete the treatment course. Similarly, the relapse rate was significantly lower in AIP patients with steroid treatment (28.6%) than in those without steroid treatment (42.5%). Steroid re-treatment was effective in all relapsed patients with or without steroid therapy.

CONCLUSION: Steroid therapy should be considered in all patients with active inflammatory phase of AIP. However, the optimal regimen still should be trailed in larger numbers of patients with AIP.

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Key words: Autoimmune pancreatitis; Chinese population; Steroid therapy; Remission; Relapse

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INTRODUCTION

Autoimmune pancreatitis (AIP) has recently been described as a type of chronic pancreatitis characterized by an autoimmune inflammatory process with prominent lymphocyte infiltration[1]. Since the concept of autoimmune pancreatitis was firstly introduced by Yoshida *et al*[2]

in 1995, the number of AIP reports has been recently increased in the medical literature.

AIP is characterized by diffuse or focal enlargement of the pancreas, irregular narrowing of the main pancreatic duct, elevated serum immunoglobulin G4 (IgG4) and presence of autoantibodies, and/or IgG4-positive plasma cells and/or dense lymphocyte infiltration with fibrosis[3]. Before the availability of the diagnostic criteria of AIP proposed by the Japan Pancreas Society[4], AIP was frequently misdiagnosed as pancreatic cancer, which consequently imposed the patients a superfluous pancreatic resection[5].

Since the fibroinflammatory process of AIP has a favorable response to steroids, steroid therapy has been accepted as a standard treatment for AIP[6]. Treatment protocols for AIP are still evolving. Although the initial and maintenance dose of oral steroids are clearly recommend by the Japanese literature[7], there is little consensus worldwide on a steroid treatment regimen for patients with AIP[8]. Hence, to explore the optimal steroid therapeutic strategy for AIP, we conducted a retrospective study on AIP treatment in two large institutions in China.

MATERIALS AND METHODS

Subjects

This retrospective study was done to evaluate steroid therapeutic strategy for patients with AIP. The study was conducted in the General Hospital of Tianjin Medical University and West China Hospital of Sichuan University. A total of 68 patients with clinically, radiologically and biochemically diagnosed AIP were enrolled.

Diagnostic criteria

AIP was diagnosed according to the Asian diagnostic criteria for AIP[9]. Consequently, the diagnosis was based on the following clinicopathological findings: (1) the imaging criteria including diffuse/segmental/focal enlargement of the pancreas and diffuse/segmental/focal pancreatic ductal narrowing, often with the stenosis of the bile duct; (2) the serological criteria including elevated serum IgG or IgG4 levels, or detection of autoantibodies; and (3) and/or the histopathological criteria including lymphoplasmacytic infiltration and fibrosis, with abundant IgG4-positive cell infiltration. Thus, AIP was diagnosed when the imaging criteria and one of the other two criteria, the serological and histopathological, were satisfied. Optionally, AIP can also be diagnosed with fulfillment of both the imaging criteria and a good response to steroid treatment.

Assessment of the investigations and therapeutic strategy

We assessed the performed radiological investigations and biochemical tests for all AIP patients. We analyzed remission and relapse whether with or without steroid treatment as well as the given steroid treatment regimen in the patients who received steroid treatment. Remission was defined as the disappearance of clinical symptoms and resolution of the pancreatic and/or extrapancreatic manifestations on radiological investigations[10-12]. All the patients underwent periodic laboratory tests and imaging studies every 3 mo in the first year after remission resulting from steroid treatment. Relapse was defined as reappearance of symptoms with the development of pancreatic and/or extrapancreatic abnormalities on imaging studies and/or marked elevation of serum IgG or IgG4 levels[11,12]. Relapse also included re-elevation of serological levels alone exclusive of clinical symptoms or abnormal imaging[7].

Statistical analysis

Statistical analysis was performed using Fisher's exact test and Mann-Whitney's *U* test (with commercially available software SPSS version 13.0; SPSS, Inc, Chicago, IL). Data were expressed as mean ± SD or median (range). The period from the start of steroid treatment to relapse was evaluated using the Kaplan-Meier method. Differences with P values < 0.05 were considered significant.

RESULTS

Patient profile

Sixty-eight patients with AIP fulfilling the Asian diagnostic criteria for AIP were included in this study. There were 50 men and 18 women with an average age of 62.5 years [patients with steroid 62 (43-78) years, patients without steroid 64 (47-72) years]. Forty patients underwent various surgical procedures (30 males, 10 females), while the remaining 28 patients received steroid treatment (20 males, 8 females). The surgical procedures included pancreatoduodenectomy, distal pancreatectomy and choledochojejunostomy, radiofrequency ablation for the enlarged pancreatic head, percutaneous transhepatic biliary drainage and endoscopic biliary drainage. The numbers of patients undergoing various surgical procedures are as follow: (1) thirteen underwent pancreatoduodenectomy; (2) nineteen underwent distal pancreatectomy and choledochojejunostomy; (3) three underwent radiofrequency ablation for the enlarged pancreatic head; (4) three underwent percutaneous transhepatic biliary drainage; and (5) two underwent endoscopic biliary drainage. In the current study, no patient received any other immunosuppressive treatments such as azathioprine or ursodeoxycholic acid or mycophenolate mofetil.

Given steroid therapy

Steroid therapy was administered in 28 of the 68 AIP patients who were initially presented with obstructive jaundice, abdominal pain, rapid weight loss (> 5 kg in the past 3 mo), diffuse enlargement of the pancreas, associated extrapancreatic abnormalities such as cholangitis and retroperitoneal fibrosis. The clinical presentations in the 28 patients are shown in Table 1.

Table 1 Clinical presentation of patients with steroid treatment *n* (%)

Clinical presentation	Patients (*n* = 28)
Obstructive jaundice	17 (60.7)
Abdominal pain	9 (32.1)
Rapid weight loss	6 (21.4)
Diffuse enlargement of the pancreas	17 (60.7)
Associated extrapancreatic abnormalities	
Cholangitis	18 (64.3)
Retroperitoneal fibrosis	3 (10.7)

Before steroid therapy, blood glucose levels were controlled using insulin in 6 (21.4%) patients and oral hypoglycemics in AIP patients with diabetes mellitus. Alternatively, for patients showing hyperbilirubinemia > 3 mg/dL, percutaneous transhepatic biliary drainage and endoscopic biliary drainage were performed in 4 and 3 patients, respectively.

Regimens of steroid therapy

Among the 28 AIP patients who received steroid therapy, 18 (64.3%) patients started on oral prednisolone at 30 mg/d, while 10 (35.7%) patients began oral prednisolone at 40 mg/d. The therapy monitoring 3 wk after starting steroid treatment was achieved based on the patient's clinical presentation as well as the biochemical results and the imaging findings. When steroid treatment was effective, the dose was tapered by 5 mg every 1-2 wk until the dose reached a maintenance dose of 5 mg/d. Then, maintenance steroids for at least 6-12 mo were given to complete the treatment course. The maintenance steroid treatment was withdrawn whenever complete radiological and serological improvement was obtained.

Remission rate with and without steroid therapy

Steroid therapy used in this study was very effective in alleviating the clinical presentation of AIP (such as obstructive jaundice and abdominal pain) and induced remission more quickly than other treatments without steroids. Likewise, clinical and radiological responses to steroid therapy were seen in 2-3 wk in this study. We also found that response to steroid therapy was typically rapid with significant radiological improvement at 2-3 wk.

The remission rate of AIP patients with steroid treatment (27/28 patients, 96.4%) was significantly higher ($P < 0.001$) than in those without steroid treatment (30/40 patients, 75%). Furthermore, the period to yield a remission in the patients treated with an initial prednisolone dose of 30 mg/d was not significantly different ($P = 0.273$) from the period in those treated with an initial prednisolone dose of 40 mg/d (6.4 ± 5.72 mo and 6.1 ± 6.05 mo, respectively). At remission, the enlarged pancreas returned to near-normal size in 22 (78.6%) patients. Pancreatic atrophy occurred in 6 (21.4%) patients and persistent focal enlargement was found in 1 (3.6%) patient. Irregularity of the pancreatic ducts and/or some degree of bile duct stenosis remained in 15 (53.6%) patients. Although the elevated serum IgG level declined in all patients after the start of steroid treatment, it failed to normalize (< 751 mg/dL) in 17 (60.7%) patients. Likewise, 15 (53.6%) patients with persistently elevated serum IgG levels showed irregular pancreatic ducts and/or some degree of bile duct stenosis.

Relapse rate with and without steroid therapy

The relapse rate was significantly lower ($P = 0.004$) in AIP patients with steroid treatment than in those without steroid treatment (8/28 patients, 28.6% and 17/40 patients, 42.5%, respectively). The relapse period after starting steroid treatment differed in the 8 patients: within 6 mo in 1 (12.5%) patient, within 1 year in 4 (50%) patients, within 2 years in 6 (75%) patients and within 3 years in all 8 (100%) patients. Multivariate analysis showed that there was no correlation ($P = 0.573$) between the relapse rate and the initial prednisolone dose (20% with prednisolone dose of 40 mg/d and 22.2% with prednisolone dose of 30 mg/d). Furthermore, the relapse occurred in 2 of the 10 patients who received prednisolone at 40 mg/d and in 4 of the 18 patients who received prednisolone at 30 mg/d. The relapse rate of AIP was significantly higher ($P = 0.003$) in patients with persistently elevated serum IgG levels (6/17 patients, 35.3%) than in those with normalized serum IgG levels (1/7 patients, 10%). Relapse occurred in 3 of the 8 (37.5%) patients during the maintenance treatment, whereas it occurred in the rest of the 5 (62.5%) patients when the maintenance treatment was stopped. Steroid re-treatment was effective in all relapsed patients with or without steroid therapy.

Maintenance dose of oral steroids after remission

The maintenance steroid therapy was administered after remission in all 28 AIP patients treated with steroids. After remission, the starting oral prednisolone dose was gradually tapered by 5 mg every 1-2 wk to reach a maintenance dose of 5 mg/d. The maintenance steroids were continued for at least 6-12 mo to complete the treatment course. The maintenance dose of steroid was withdrawn in 7 (25%) patients in whom complete radiological and serological improvement was obtained.

Complications of steroid treatment

There was no death attributable to steroid treatment related complications. As AIP patients were typically elder patients, they were at a high risk of developing steroid related complications. Steroid treatment related complications occurred in 7 of the 28 patients, including pneumonia in 3 patients, avascular necrosis of the femoral head in 1 patient, lumbar vertebral fracture in 1 patient and diabetes mellitus in 2 patients. In these patients, the steroid medication dose was reduced or completely ceased. In addition, the 3 patients with pneumonia were treated with antibiotics.

DISCUSSION

It has been reported that the overall prevalence of AIP was 0.82/100 000 in Japan. Moreover, on the basis of clinical presentation, biochemical results or histological findings, AIP constitutes 5%-11% of all patients with chronic pancreatitis[13,14]. Likewise, Song *et al*[15] stated that AIP patients accounted for 5% of chronic pancreatitis population in China.

In this study, AIP was found predominantly in elderly males. This coincides with the feature that although AIP occurs in both sexes, it is at least twice as common in men as in women. In addition, in spite that AIP varies widely in age, most AIP patients are older than 50 years[16]. The clinical presentation of AIP in the current study was in agreement with the studies by Kim *et al*[12] and Nishimori *et al*[17]. AIP was presented with obstructive jaundice in 49 (72.1%) patients, abdominal pain in 30 (44.1%) patients and rapid body weight loss in 21 (30.9%) patients. Alternatively, diabetes mellitus was present in 40 (58.8%) patients.

Moreover, AIP has frequently been diagnosed or suspected as pancreaticobiliary malignancy, which consequently imposed the patients an unnecessary pancreatic resection[18,19]. This study also showed that 40 (58.8%) patients with AIP underwent unnecessary surgical procedures.

Alternatively, Refaat *et al*[20] showed in their case report of AIP that although the imaging findings were suggestive of AIP, pancreatic biopsy was necessary to differentiate AIP from pancreatic cancer as the management differs significantly. Consequently, clinicians should be conversant with the clinical, radiographic, serologic and histologic evidence of AIP to improve the diagnostic accuracy for AIP.

Although steroid therapy is generally considered to be very effective in the initial inflammatory phase of AIP, its facile use for patients whose diagnosis of AIP is questionable should be prohibited[21]. In this study, steroid therapy was very effective in improving patient's clinical presentation such as obstructive jaundice and abdominal pain. Additionally, it induced remission more quickly than surgical treatments as the clinical presentation, the imaging findings and the laboratory results were improved in all cases in four week after steroid treatment. This result concurs with Hirano *et al*[22]. Therefore, we think that steroid therapy should be considered in all patients with AIP with active disease. This is in agreement with the study by Pannala *et al*[8].

There has been no consensus to date on steroid regimen and duration of treatment in AIP. Mayo Clinic recommended the initial oral prednisolone dose of 40 mg/d for 4 wk. Afterward, laboratory tests and radiological investigation should be performed 4-6 wk after initiating treatment. If there are biochemical and radiological responses, the dose is gradually tapered by 5 mg every week to complete the treatment course of 11 wk[10]. Considering that many complications are related to steroid treatment, maintenance low-dose oral prednisolone treatment was not routinely performed for patients with AIP[8]. In a Japanese consensus statement, Kamisawa *et al*[7] suggested that initiating treatment with prednisolone at 0.6 mg/kg daily should be tapered gradually to reach a maintenance dose of 5 mg/d over 3-6 mo. Finally, maintenance steroids are continued for at least 6 mo and possibly up to 3 years. Although the remission can be achieved in most AIP patients after steroid treatment[9], relapses still occur in 24%-40% of the patients and the starting doses of steroid (typically given for 3-4 wk) are often continued for varying periods of time[8].

In our practice, the starting oral prednisolone dose was 30-40 mg/d for 3 wk. Then, we monitored the patient's clinical presentation, the biochemical and serological results as well as the imaging findings in 3 wk after starting steroid treatment. Consequently, when steroid treatment was effective, we tapered the dose by 5 mg every 1-2 wk to reach a maintenance dose of 5 mg/d. Lastly, maintenance steroids were continued for at least 6-12 mo to complete the given treatment course. The maintenance steroid treatment was withdrawn once complete radiological and serological improvement was achieved. In this study, the response was typically rapid through significant radiological improvement at 2-3 wk in 96.4% of the patients. This coincides with Kamisawa *et al*[23].

Future studies should help to establish an optimal steroid therapy regimen to effectively alleviate the clinical presentation of AIP patients and reduce the risk of steroid-induced complications.

Similar to the previous studies[6,7], we also found that AIP patients were typically elder patients and are at a high risk of developing steroid related complications (diabetes, pneumonia and so forth). Instead of using long-term low-dose steroids, azathioprine, mycophenolate mofetil and other immunosuppresive drugs are used for maintenance of remission in AIP patients who relapse after steroid withdrawal[10]. But, similar to steroids, azathioprine or mycophenolate mofetil also have significant side effects such as increased risk of infection, headache, peripheral edema, hypertension, leucopoenia and lymphoma[8,24]. Thus, the option of long-term treatment for maintenance of AIP remission needs prospective and controlled trials in larger numbers of patients to assess the risk to benefit ratio of each approach.

In conclusion, steroid therapy, usually in a dose range of 30-40 mg/d, is effective in AIP patients and should be considered for all AIP patients in the acute inflammatory phase. The optimal regimen of steroid therapy remains to be determined.

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COMMENTS

Background

There is no consensus so far on steroid regimen for patients with autoimmune pancreatitis (AIP).

Research frontiers

Steroid therapy has been accepted as a standard treatment for AIP. But, there is little consensus worldwide on a steroid treatment regimen for patients with AIP.

Innovations and breakthroughs

This is a retrospective study exploring the optimal steroid therapeutic strategy for AIP. The performed radiological investigations and biochemical tests, the regimen of the given steroid treatment, remission and relapse whether with and without steroid therapy were analyzed.

Applications

The study results suggest that steroid therapy should be considered in all patients with active inflammatory phase of AIP.

Terminology

Remission: the disappearance of clinical symptoms and resolution of the pancreatic and/or extrapancreatic manifestations on radiological investigations. Relapse: reappearance of symptoms with the development of pancreatic and/or extrapancreatic abnormalities on imaging studies and/or marked elevation of serum immunoglobulin G (IgG) or IgG4 levels

Peer review

This is a good retrospective study in which authors analyzed the optimal steroid therapeutic strategy for AIP. The results are interesting and suggest that steroid therapy should be considered in all patients with active inflammatory phase of AIP.

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BRIEF ARTICLE

Increased expression of tyrosine phosphatase SHP-2 in *Helicobacter pylori*-infected gastric cancer

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Abstract

AIM: To explore the alteration of tyrosine phosphatase SHP-2 protein expression in gastric cancer and to assess its prognostic values.

METHODS: Three hundred and five consecutive cases of gastric cancer were enrolled into this study. SHP-2 expression was carried out in 305 gastric cancer specimens, of which 83 were paired adjacent normal gastric mucus samples, using a tissue microarray immunohistochemical method. Correlations were analyzed between expression levels of SHP-2 protein and tumor parameters or clinical outcomes. Serum anti-*Helicobacter pylori* (*H. pylori*) immunoglobulin G was detected with enzyme-linked immunosorbent assay. Cox proportional hazards model was used to evaluate prognostic values by compassion of the expression levels of SHP-2 and disease-specific survivals in patients.

RESULTS: SHP-2 staining was found diffuse mainly in the cytoplasm and the weak staining was also observed in the nucleus in gastric mucosa cells. Thirty-two point five percent of normal epithelial specimen and 62.6% of gastric cancer specimen were identified to stain with SHP-2 antibody positively ($P < 0.001$). Though SHP-2 staining intensities were stronger in the *H. pylori* (+) group than in the *H. pylori* (-) group, no statistically significant difference was found in the expression levels of SHP-2 between *H. pylori* (+) and *H. pylori* (-) gastric cancer ($P = 0.40$). The SHP-2 expression in gastric cancer was not significantly associated with cancer stages, lymph node metastases, and distant metastasis of the tumors ($P = 0.34$, $P = 0.17$, $P = 0.52$). Multivariate analysis demonstrated no correlation between SHP-2 expression and disease-free survival ($P = 0.86$).

CONCLUSION: Increased expression of SHP-2 protein in gastric cancer specimen suggesting the aberrant up-regulation of SHP-2 protein might play an important role in the gastric carcinogenesis.

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Key words: Gastric cancer; SH2-containing protein tyrosine phosphatase 2; Expression; *Helicobacter pylori*

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INTRODUCTION

Gastric cancer remains the fourth most commonly diagnosed cancer and is the second leading cause of cancer related deaths worldwide[1]. The SHP-2 tyrosine posphatase encoded by tyrosine phosphatase SHP-2 (PTPN11) is an evolutionarily conserved protein, containing two Src homology domains at the N terminus, a central catalytic domain, and a C-terminal tail[2,3]. SHP-2 positively regulates many signaling pathways, such as insulin, epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, interleukin (IL)-1, and IL-6[4-6]. SHP-2 has been identified as an essential component in several oncogenic signaling pathways. Elucidation of the events underlying SHP-2-evoked transformation may provide new insights into oncogenic mechanisms and novel targets for anti-cancer therapy[7-11]. Meanwhile, infection with *Helicobacter pylori* (*H. pylori*) is the leading cause for developing atrophic gastritis and gastric cancer worldwide. Many studies reveal that strains of *H. pylori* carrying the major protein virulence factor, cytotoxin-associated antigen A (CagA), are associated with increased risks of gastric cancer compared to strains of *H. pylori* lacking CagA[12]. *H. pylori* inject CagA protein into gastric epithelial cells *via* the bacterial type Ⅳ secretion system and then CagA localizes to the cell membrane and aberrantly activates SHP-2 and its downstream effectors. However, little effort has been devoted to assess an association of the expression level of SHP-2 with gastric cancer risk[13-15]. To investigate the relationship between *H. pylori* infection and SHP-2 protein production in gastric cancer, the SHP-2 protein expression was investigated in 305 patients with gastric cancer, and paired adjacent normal tissue samples were collected in 83 patients. Associations of the protein expression with patient clinical characteristics and prognostic values were also explored in this study.

MATERIALS AND METHODS

Patients and tissue specimens

Three hundred and five consecutive cases of gastric cancer were enrolled in the study. The patient did not receive any treatment before the surgical operation. Gastric cancers were removed by surgical excisions. Adjacent normal gastric epithelial samples were also collected from 83 patients for comparison. Patient ages ranged from 32 to 87 years, with a median age of 64 years. The diagnosis of gastric cancer was confirmed by two pathologists (Jin MS, Wang YP) independently at First Hospital of Jilin University. Written informed consent was obtained from each patient and the study protocol was approved by the Ethics Committee of the First Hospital of Jilin University.

Immunohistochemistry

The section (4 μm in width) of the archival paraffin-embedded block was excised, deparaffinized and stained using a streptavidin-biotin immunoperoxidase technique. The primary antibody, anti-SHP-2 monoclonal antibody (Santa Cruz Biotec, United States), was used in 1:500 dilution; and 3, 3-diaminobenzidine was employed as a chromogen. The section was counterstained with hematoxylin. The stained slides were evaluated by two independent pathologists (Jin MS, Wang YP), who were blinded to the clinical data. The widely accepted H-score system was used to assess staining intensity and percentage of the cells stained with a specific magnitude of intensity. The H-score was calculated with the following equation: H-score = $\sum$Pi (i) (i = 0, 1, 2, 3, Pi = 0%-100%), where i means the intensity of staining, 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. and Pi represents percentages of stained cells with intensities varying from 0% to 100%. Therefore, the H-score ranged from 0 to 300. The H-score $\geqslant$ 100 is considered as positive staining and < 100 is considered as negative staining.

Determination of H.pylori infection

Among 305 gastric cancer patients, blood samples were collected from 100 patients for the examination of *H. pylori* infection before the surgical operation. Four mL of fasting blood samples were left at room temperature for 30 min and the centrifuged. The serum was drawn and stored at a -80 ℃ freezer. Serum immunoglobulin G (IgG) antibodies to *H. pylori* were detected by *H. pylori*-IgG enzyme-linked immunosorbent assay kit (Biohit, Helsink, Finland). The antibody titers were defined by optical density values according to the manufacturer's protocol, and titers higher than the cut off value of 30EIU were considered as positive for *H. pylori* infection. The kit quality control samples showed CVs of 4.5% for *H. pylori* infection examination.

Statistical analysis

As SHP-2 H-scores and their difference values were not normally distributed, these continuous variables were presented as median (interquartile). The Wilcoxon matched-pairs signed-rank test and Wilcoxon signed-rank test were used when comparing paired groups and two independent groups, respectively. Disease-specific survival analysis was performed using the Kaplan-Meier method with log rank test. Risk ratios and corresponding 95% CIs were calculated by the Cox proportional hazards model after adjusted by age (scale variable), sex (nominal variable), differentiation (nominal variable), lymph-vascular invasion (nominal variable) and tumor node metastasis (TNM) staging (scale variable). All statistical tests were two-tailed and *P* values < 0.05 were considered statistically significant. All analyses were performed using the SPSS software package 18.0 (SPSS Inc., United States).

RESULTS

Expression of SHP-2 in gastric cancer specimen and normal gastric epithelial tissues

SHP-2 staining was found diffuse mainly in the cytoplasm and the weak staining was also observed in the nucleus (Figure 1). SHP-2 positive staining was found in

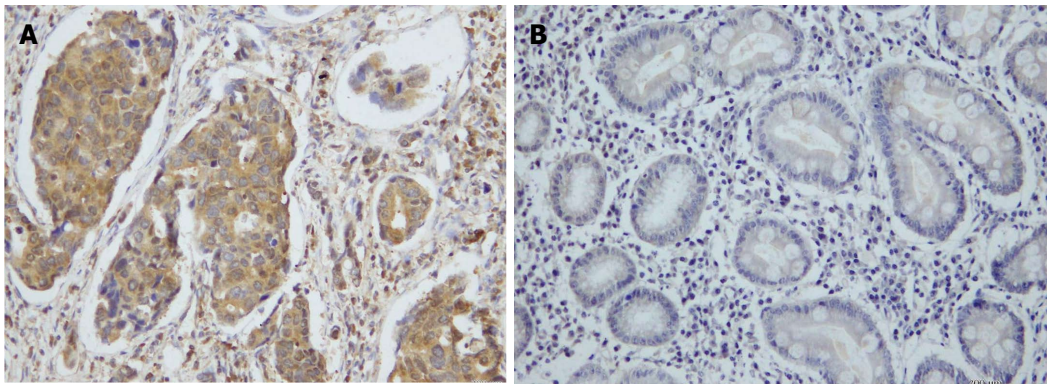

Figure 1 Visualization of SHP-2 expression in malignant gastric epithelial cells and normal mucosal cells by immunohistochemistry (× 200). A: SHP-2 positive immunostaining (2+) in the cytoplasm of gastric carcinoma cells; B: SHP-2 negative immunostaining in glandular cells of normal gastric mucosa.

Table 1 SHP-2 expression in 83 paired gastric cancers and adjacent normal mucus and SHP-2 by *Helicobacter pylori* infection in gastric cancer groups *n* (%)

	H-score				Median H-score (quartile)	*P* value
	0	1-99	100-200	201-300		
Cancer (n = 83)	10 (12.0)	21 (25.3)	27 (32.5)	25 (30.1)	160 (40-210)	< 0.001
Control (n = 83)	41 (49.4)	15 (18.1)	21 (25.3)	6 (7.2)	20 (0-160)	
Positive (n = 67)	8 (11.9)	8 (11.9)	24 (35.8)	27 (40.3)	180 (120-270)	0.401
Negative (n = 33)	1 (3.0)	10 (30.3)	11 (33.3)	11 (33.3)	160 (85-240)	

62/83 (62.6%) gastric cancer samples and in basal cells of 27/83 (32.5%) normal mucosal samples, respectively. There was a significantly increased rate of SHP-2 positive expression in gastric cancer compared to the normal mucosa ($P < 0.001$). In addition, the results showed statistically significant difference in the median H-score of SHP-2 expression between the cancer lesion and normal mucosa (160 *vs* 20, $P < 0.001$; Table 1).

SHP-2 levels and H. pylori infection

Among 100 gastric cancer patients tested for *H. pylori* antibodies, the positive rate of *H. pylori* infection was 67.0%. In the age subgroup analysis, the *H. pylori* infection rate was higher in the group aged between 45-65 years compared to the groups aged younger than 45 years, or older than 65 years. The results showed no correlation between *H. pylori* infection and the positive staining rate of SHP-2 expression ($P = 0.40$). Though SHP-2 staining intensities were stronger in the *H. pylori* (+) group than in the *H. pylori* (-) group, no statistical difference was observed between the two groups (76.1% *vs* 66.6%, $P = 0.09$; Table 1).

SHP-2 expression and clinical characteristics

Statistically, the SHP-2 expression in gastric cancer was not associated with the tumor stage, lymph node metastasis and distant metastasis of the tumors ($P = 0.34$, $P = 0.17$, $P = 0.52$). Kaplan-Meier survival curve was plotted for the 305 patients according to H-score of SHP-2, the overall survival rates of the four groups was not significantly different (log rank test, $P = 0.86$; Figure 2). In addition, the multivariate analysis did not demonstrate an association between SHP-2 expression and disease specific death. There were no differences of SHP-2 levels, age, sex, tumor differentiation, lymph-vascular invasion, TNM staging and survival time between subgroups (83 and 100 cases) and total subjects. The clinical characteristics of subjects are summarized in Tables 2 and 3.

DISCUSSION

Tyrosine phosphorylation and dephosphorylation are coordinated by tyrosine kinases and phosphatases. Recently, SHP-2 has been identified to play a vital role in the pathogenesis of human cancers[10,16,17]. Over-expressions of SHP-2 were reported in infiltrating ductal carcinomas of breast and in gastric cancers as well,[16,18] suggesting that the up-regulation of SHP-2 protein is involved in developing these cancers. In addition, the SHP-2 expression in breast cancers was positively correlated with lymph node metastases and tumor grades, implying that SHP-2 expression is associated with the progression of breast cancers[16]. By contrast, SHP-2 expression in gastric cancers was not found to be associated with lymph node metastases, neither tumor grades in our study (Table 2). The down-regulation in SHP-2 expression was identified to be a better prognostic marker in patients with hepatocellular carcinoma, indicating that SHP-2 plays a role in the tissue specific manner[10].

In general, SHP2 plays multiple roles in tumorigenesis and immune responses[7,8,12]. Activated glycogen synthase kinase-3β, bioactive lipids and their enzymatic generators have been reported to synergistically facilitate inter-

Table 2 SHP-2 expressions and clinical characteristics of 305 gastric cancer patients

	H-score, *n* (%)				Median H-score (quartile)	*P* value
	0	1-99	100-200	201-300		
Sex						
Male (*n* = 233)	16 (6.9)	44 (18.9)	79 (33.9)	94 (40.3)	160 (100-240)	0.82
Female (*n* = 72)	4 (5.6)	8 (11.1)	36 (50.0)	24 (33.3)	160 (140-240)	
Age						
≤ 60 yr (*n* = 129)	9 (7.0)	23 (17.8)	50 (38.8)	47 (36.4)	160 (110-240)	0.92
> 60 yr (*n* = 176)	11 (6.3)	29 (16.5)	65 (36.9)	71 (40.3)	180 (110-240)	
Differentiation						
Well (*n* = 6)	0 (0)	1 (16.7)	1 (16.7)	4 (66.7)	225 (155-270)	0.43
Moderate (*n* = 114)	8 (7.0)	20 (17.5)	41 (36.0)	45 (39.5)	160 (115-240)	
Poor (*n* = 185)	12 (6.5)	31 (16.8)	73 (39.5)	69 (37.3)	160 (120-240)	
Lymph-vascular invasion						
No (*n* = 142)	9 (6.3)	22 (15.5)	54 (38.0)	57 (40.1)	160 (120-240)	0.89
Yes (*n* = 163)	11 (6.7)	30 (18.4)	61 (37.4)	61 (37.4)	160 (100-240)	
Nerve invasion						
No (*n* = 184)	14 (7.6)	30 (16.3)	70 (38.0)	70 (38.0)	160 (120-240)	0.40
Yes (*n* = 121)	6 (5.0)	22 (18.2)	45 (37.2)	48 (39.7)	160 (120-240)	
TNM staging						
Ⅰ (*n* = 25)	0 (0)	3 (12.0)	12 (48.0)	10 (40.0)	180 (140-270)	0.34
Ⅱ (*n* = 50)	4 (8.0)	8 (16.0)	13 (26.0)	25 (50.0)	195 (115-270)	
Ⅲ (*n* = 194)	14 (7.2)	32 (16.5)	77 (39.7)	71 (36.6)	160 (120-240)	
Ⅳ (*n* = 36)	0 (0)	7 (20.6)	14 (41.2)	13 (38.2)	160 (100-242)	
Invasion						
T1 (*n* = 9)	0 (0)	0 (0)	4 (44.4)	5 (55.6)	240 (170-270)	0.34
T2 (*n* = 37)	1 (2.7)	7 (18.9)	18 (48.6)	11 (29.7)	160 (120-225)	
T3 (*n* = 225)	19 (8.4)	38 (16.9)	79 (35.1)	89 (39.6)	160 (100-240)	
T4 (*n* = 34)	0 (0)	7 (20.6)	14 (41.2)	13 (38.2)	160 (120-248)	
Lymph node metastases						
N0 (*n* = 66)	3 (4.5)	10 (15.2)	23 (34.8)	30 (45.5)	180 (140-270)	0.17
N1 (*n* = 94)	7 (7.4)	15 (16.0)	31 (33.0)	41 (43.6)	180 (120-240)	
N2 (*n* = 79)	3 (3.8)	15 (19.0)	39 (49.4)	22 (27.8)	160 (120-210)	
N3 (*n* = 66)	7 (10.6)	12 (18.2)	22 (33.3)	25 (37.9)	160 (100-270)	
Distant metastasis						
No (*n* = 266)	18 (6.8)	43 (16.2)	101(38.0)	104 (39.1)	170 (120-240)	0.52
Yes (*n* = 39)	2 (5.1)	9 (23.1)	14 (35.9)	14 (35.9)	160 (100-240)	
Survival						
Survived (*n* = 180)	12 (6.7)	28 (15.6)	69 (38.3)	71 (39.4)	180 (120-240)	0.77
Died (*n* = 125)	8 (6.4)	24 (19.2)	46 (36.8)	47 (37.6)	160 (100-240)	

TNM: Tumor node metastasis.

Table 3 Multivariate analyses for disease-specific survival

	RR (95% CI)	*P* value
SHP-2 expression		
H-score (201-300) (*n* = 118)		
H-score (100-200) (*n* = 115)	0.99 (0.63-1.56)	0.98
H-score (1-99) (*n* = 52)	1.08 (0.72-1.64)	0.70
Negative (0) (*n* = 20)	1.04 (0.49-1.67)	0.82
Tumor node metastasis staging		
Ⅰ (*n* = 25)		
Ⅱ (*n* = 51)	1.87 (0.52-6.79)	0.34
Ⅲ (*n* = 195)	3.94 (1.22-12.74)	0.02
Ⅳ (*n* = 34)	13.63 (4.04-46.06)	0.00
Lymph-vascular invasion		
Yes (*n* = 142)		
No (*n* = 163)	1.61 (1.09-2.37)	0.02

Calculated by age, sex, differentiation, lymph-vascular invasion and tumor node metastasis staging adjusted Cox regression model. RR: Relative risk.

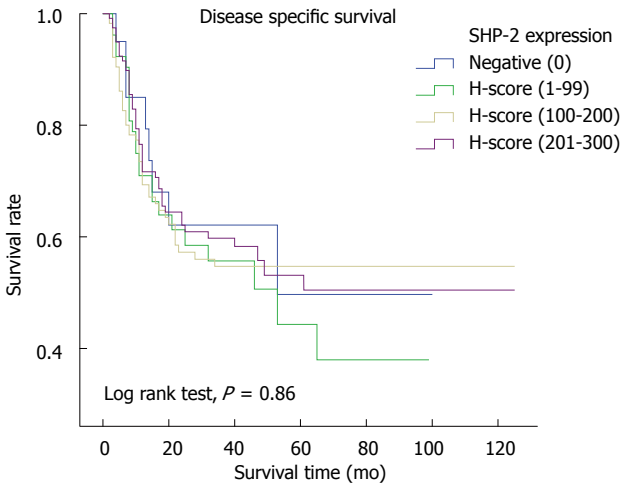

Figure 2 SHP-2 expression and disease specific survival.

ferons (IFN)-γ-induced STAT1 activation by inhibiting SHP-2[19]. Increasing evidences demonstrate that SHP-2 regulates immune responses through its effects on cytokine signaling pathways or inhibits receptor signaling

pathways, such as IFN, tumor necrosis factor-alpha, and JAK/STAT pathways[20-22]. These results support a hypothesis that increased SHP-2 expression would lead to changes in immune responses, eventually resulting in malignant transformation.

H. pylori is a group I carcinogen according to the World Health Organization and International Agency for Research on Cancer[23]. It has been generally accepted that gastric cancer is a multistep process involving a series of events such as atrophic gastritis, intestinal metaplasia, dysplasia and carcinoma. Several studies demonstrated that CagA molecules tethered to the inner surface of plasma membrane of gastric epithelial cells, where they are tyrosine phosphorylated[14,24-26]. SHP-2 is specifically bound by tyrosine phosphorylated CagA, which provokes RAS- and extracellular signal-regulated kinase-dependent signaling cascades. The deregulation of SHP-2 signal transductions leads to loss of epithelial cell polarity, manifested by cell elongation and increased motility, that has been considered to be an important mechanism by which CagA-positive *H. pylori* promotes gastric carcinogenesis[14]. Recently, we reported that rs12423190 polymorphism of the *PTPN11* gene (encoding SHP-2 protein) is significantly associated with an increased risk of gastric atrophy in *H. pylori* infection[27]. To the best of our knowledge, the present study is the first clinical evaluation of SHP-2 expression levels and *H. pylori* infection in gastric cancer. Although SHP-2 expression level was higher in the *H. pylori* (+) group of gastric cancer compared to the *H. pylori* (-) group, no statistical difference was found (76.1% *vs* 66.6%, $P = 0.40$).

In conclusion, we demonstrated that SHP-2 is overexpressed in gastric cancer. However, the level of SHP2 expression was associated with neither *H. pylori* infection nor prognosis of the tumor. Targeting SHP-2 might lead to development of a novel treatment for gastric cancer. The precise mechanisms underlying the effect of SHP-2 on gastric carcinogenesis remains to be further investigated[28-31].

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COMMENTS

Background

Gastric cancer remains the second leading cause of cancer-related deaths worldwide. Recently, SHP-2 has been identified to play a vital role in the pathogenesis of human cancers.

Research frontiers

SHP-2 has been identified as an essential component of several oncogenic signaling pathways. Mutation in SHP-2 has been confirmed in several types of solid tumors. Elucidation of the events underlying SHP-2-evoked transformation may provide new insights into the novel targets for anti-cancer therapy.

Innovations and breakthroughs

The present study is the first clinical evaluation of SHP-2 expression levels and *Helicobacter pylori* infection in gastric cancer patients.

Applications

Targeting SHP-2 might lead to development of a novel treatment for gastric cancer in the future, such as SHP-2 inhibitors.

Terminology

SHP-2 tyrosine posphatase encoded by tyrosine phosphatase SHP-2 is an evolutionarily conserved protein, containing two Src homology domains at the N terminus, a central catalytic domain, and a C-terminal tail. SHP-2 positively regulates many signaling pathways.

Peer review

The authors showed that SHP-2 expression was higher in cancer specimen compared with normal specimen. However, no association was found between SHP-2 and other clinical parameters.

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CASE REPORT

Acute hepatitis C virus infection in a nurse trainee following a needlestick injury

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Abstract

Hepatitis C virus (HCV) infection after biological accident (needlestick injury) is a rare event. This report describes the first case of acute HCV infection after a needlestick injury in a female nursing student at Padua University Hospital. The student nurse was injured on the second finger of the right hand when recapping a 23-gauge needle after taking a blood sample. The patient who was the source was a 72-year-old female with weakly positive anti-HCV test results. Three months after the injury, at the second step of follow-up, a relevant increase in transaminases with a low viral replication activity (350 IU/mL) was observed in the student, indicating HCV infection. The patient tested positive for the same genotype (1b) of HCV as the injured student. A rapid decline in transaminases, which was not accompanied by viral clearance, and persistently positive HCV-RNA was described 1 mo later. Six months after testing positive for HCV, the student was treated with pegylated interferon plus ribavirin for 24 wk. A rapid virological response was observed after 4 wk of treatment, and a sustained virological response (SVR) was evident 6 mo after therapy withdrawal, confirming that the patient was definitively cured. Despite the favourable *IL28B* gene (rs12979860) CC- polymorphism observed in the patient, which is usually predictive of a spontaneous clearance and SVR, spontaneous viral clearance did not take place; however, infection with this genotype was promising for a sustained virological response after therapy.

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Key words: Biological risk; Acute hepatitis; Hepatitis C infection; Occupational exposure; Antiviral therapy

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INTRODUCTION

The incidence rate of acute hepatitis C virus (HCV) infection has been dramatically reduced since the advent of blood products screening and the introduction of single-use medical equipment. However, acute hepatitis C is still present in Western countries. In Italy, the incidence ranges from 1 to 14 cases per 100 000 according to the National Surveillance Agency[1], the Italian Blood Program[2] and by evaluation in the general population[3].

Currently, intravenous (IV) drug use is the most common exposure leading to HCV infection, with HCV prevalence rates of 50%-80% in long-term IV drug abusers. Other possible modes of acquisition are medical procedures, sexual intercourse and needlestick injuries, particularly in health care professionals[4-7]. The average rate of HCV transmission after a single needlestick exposure depends on the amount of inoculated blood, increasing to 0.9% with a hollow needle full of blood and decreas-

ing to 0.3% for conjunctival exposure[8]. In our previous experience at the Padua University Teaching Hospital no cases resulted in seroconversion[9].

In the present report, the first case of HCV seroconversion in our hospital following a needlestick injury is reported. Even though the spontaneous rate of viral clearance after acute hepatitis C ranges from 20% to 50%[5,10] and it can be predicted by measurement of serum viral load[11], which should become negative within 12-14 wk after the exposure, the injured patient developed acute hepatitis without spontaneous viral clearance. The student nurse was treated with pegylated interferon and ribavirin.

CASE REPORT

A 19-year-old female student nurse was injured on the second finger of the right hand when recapping a 23-gauge needle after taking a blood sample. The patient who was the source was a 72-year-old female, weakly positive for anti-HCV by chemiluminescence (CIA) (VITROS, anti-HCV assay, Ortho Clinical Diagnostic, Abbott Laboratories, IL, United States) with an undetermined pattern (only C1+ and C2+) at confirmatory test by immunoblot assay (HCV-RIBA 3.0 assay, Chiron Corporation, United States). Moreover, the patient had a quantitative HCV-RNA level of 14 491 571 IU/mL (COBAS TaqMan, Roche, Basel) and viral genotyping showed HCV-1b (VERSANT® HCV genotype 2.0, INNOLiPA, Innogenetics, Belgium).

The student nurse was checked and followed according to the SIROH recommendations and procedures, which consist of the assessment of hepatitis B surface antigen (HBsAg), hepatitis C antibody and human immunodeficiency virus (HIV) antibody at the time of injury (step 1), 3 mo after the injury (step 2), and 6 mo after the injury (step 3). At the time of the injury she was anti-HBsAg positive (recently vaccinated) and HIV and HCV negative. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were within the reference range.

At the second step (3 mo after injury), a high increase in transaminases with a HCV seroconversion (CIA: anti-HCV positive and RIBA: NS4+, NS3+, C1+++, C2+++, GST-) was detected in the student nurse. The student had a quantitative HCV-RNA level of 330 IU/mL and the HCV genotype was also 1b, as was that of the patient source. Despite the fact that the injured student nurse had no symptoms, she was diagnosed with acute HCV infection. Transaminases were frequently analysed, decreasing until normalization at 4 mo after the injury (Figure 1). HCV-RNA was detectable, but below the lower limit of the linearity range (< 43 IU/mL) and was negative 5 mo after injury. At this time, AST and ALT persisted within the reference range. Unfortunately, 6 mo after injury a recurrence of viraemia (HCV-RNA positive, < 43 IU/mL) together with a slight ALT spike were observed. One month later, further analysis confirmed low viral load and an increase in transaminases.

Due to the chronic profile of the infection, antiviral therapy was prescribed and the subject received a regimen consisting of 180 μg pegylated interferon α-2a subcutaneously once a week and 400 mg ribavirin (adjusted for weight), by mouth twice a day.

During this treatment (Figure 1), a persistent transaminasemia and a slight neutrophilic leukopenia were evident. HCV-RNA analysis showed that the patient remained persistently negative beginning at week 4 of therapy. The patient suffered profound fatigue and musculoskeletal pain, which showed a good response to acetaminophen. During the last month of therapy, she had severe knee pain that was resistant to acetaminophen and only partially mitigated by other non-steroidal anti-inflammatory drugs (NSAID). Therapy lasted 24 wk without any adjustment to the drug dose.

At one month after therapy withdrawal the patient had increasing asthenia and unexplained anaemia (Hb 82 g/L, reference values 123-153 g/L) that resolved with vitamins and iron supplementation, and was probably due to NSAID-induced gastrointestinal bleeding. At 6 mo, HCV-RNA analysis remained negative, transaminases remained within the reference values, the patient felt well and her blood cell counts were restored.

Annual monitoring of HCV-RNA and liver function tests confirmed viral eradication and resolution of the liver disease up to 36 mo from the onset of the infection, thus the patient was defined as "cured".

DISCUSSION

Needle recapping is an unsuitable operation, which is usually performed only by expert physicians[12]. Student nurses should only use standard precautions and procedures and the subject of this case report has been re-educated according to the protocol following her professional exposure.

Because the student had no history of blood transfusion, sexual behaviour or IV drug abuse between the time of injury and the onset of acute HCV, it was highly probable that the lesion with the contaminated source was the cause of seroconversion. Furthermore, the student nurse had the same HCV-1b genotype as the patient; however, sequence analysis comparing the source and student nurse HCV-RNA would be required to eliminate all doubt.

The average rate of HCV transmission after a single needlestick exposure is low[13], and the transmission rate in Italy is as low as 0.4%[8]. The higher seroprevalence in health care workers[2] is probably related to the fact that health care workers are submitted to follow-up after biological contamination, therefore, there is a greater rate of discovery of seroconversions than in other workers not submitted to surveillance for biological risk. The adherence to procedures after biological exposure is important, because patients with acute HCV infection are often asymptomatic and this makes diagnosis difficult and cases remain underestimated[14].

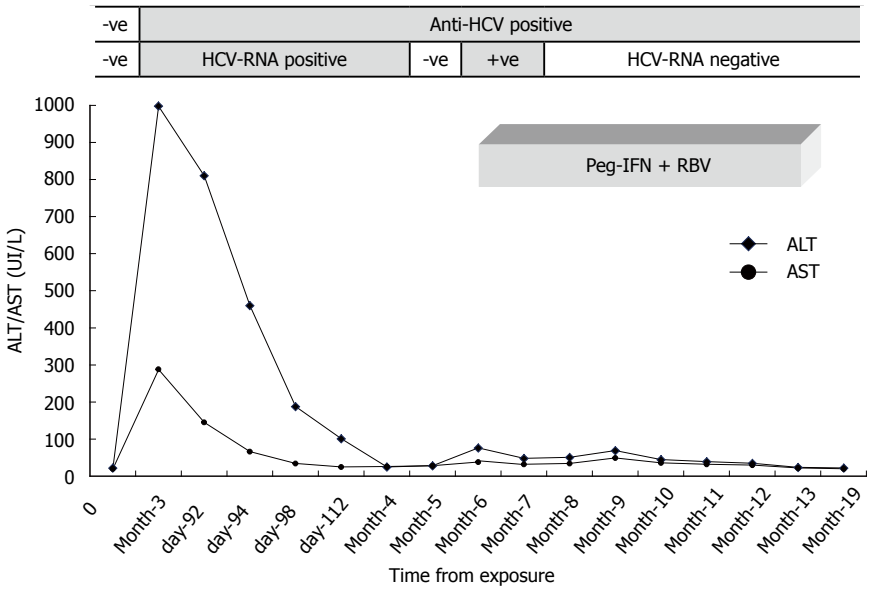

Figure 1 Behaviour of transaminases and hepatitis C virus-RNA after a needle stick injury and during the treatment with pegylated interferon α-2a at 180 μg *s.c.* once a week and ribavirin weight adjusted at 400 mg *p.o.* twice a day are shown. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HCV: Hepatitis C virus; PEG-IFN RBV: Pegylated interferon plus ribavirin.

Table 1 Immunologic mechanisms involved in acute phase of hepatitis C virus infection and predictive of viral clearance or persistence and chronicity of infection

	Immunologic marker	Mechanism involved	Ref.
Acute phase of HCV infection and predictive of viral clearance	IFN-α/-β	Vigorous IFN-stimulated genes; inhibition of IL12 expression	[21]
	IFN-γ	HCV core induced generation of reactive oxygen species with stimulation of monocytes and NKs	[22]
	IFN-λ	Host *IL28B* gene polymorphism (CC allele favorable)	[23]
	CD4/CD8	Multispecific intrahepatic Th1 T cells activation	[26]
	HLA-B27/-B57/-A3	Protective HLA alleles on T cell epitopes	[29]
HCV persistence and chronicity of infection	TGF-β/IL10	Inhibition of anti-HCV core/NS2 Th1 response	[24,25]
	CD161	HCV-specific Th17 T cells regulated by TGF-β and IL10	[27]
	HCV HVR-1 specific Abs	Viral escape from Abs mediated neutralization	[28]
	PD1/2B4/CD160	Inhibitory T cell receptors on CD8+ cells	[30]

HCV: Hepatitis C virus; HVR: Hypervariable region; IFN: Interferon; TGF-β: Transforming growth factor-β; IL: Interleukin; HLA: Human leukocyte antigen; NK: Natural killer.

The expectation of a spontaneous viral clearance was initially high in our subject due to the normalization of transaminases and the transient HCV-RNA negativity. Some factors have been proposed as being associated with spontaneous viral clearance including ethnicity[15,16], short incubation period[17], rapid decline in HCV-RNA level[11], and female gender[18]. Unfortunately, despite the age, gender, absence of additional risk factors, rapid normalization of transaminases[19], low viral load (330 IU/mL, approximately 7000 copies/mL) and favourable *IL28B* gene (rs12979860) CC-polymorphism[20] no spontaneous viral clearance was observed in our subject. This was probably due to persistence of the virus (> 3 mo from onset), and particularly to the host complex immunological mechanisms that are associated with spontaneous clearance or to chronic infection (Table 1)[21-30].

In general, following injury with a HCV source, no post-exposure prophylaxis is indicated; on the other hand, whether and how to treat acute HCV infection is still up for debate. The use of α-interferon may be effective in preventing progression from acute to chronic disease[31], but there are no data to indicate that early treatment during the course of chronic infection is less effective than immediate treatment during the acute phase. Certainly, we must consider that in 20%-30% of cases, after an acute infection, spontaneous clearance of HCV occurs and that antiviral therapy is expensive and not well tolerated in the majority of cases[32]. Delaying treatment for 12-16 wk after disease onset permits the identification of cases whose infections spontaneously resolve and whether early initiation of treatment is more appropriate in cases with genotype 1b and/or with asymptomatic disease is still an open question.

The aim of therapy is to achieve a sustained virological response (SVR), defined as the absence of HCV-RNA in serum 6 mo after therapy withdrawal[33]. In acute infection, which has the favourable features of low pretreatment HCV-RNA levels and HCV-genotype non-1 correlate,

SVR varies between 71% and 94%[34-37] and is certainly higher than the rate obtained in cases with long time chronic infection[38].

During therapy, the student nurse showed a rapid virological response (RVR), defined as the absence of HCV-RNA at 4 wk after therapy withdrawal[39], which was maintained at 6 mo after therapy withdrawal, thus, she was defined as having a SVR. These findings confirm that the earlier the virus disappears from serum the higher the probability of achieving a sustained response to treatment.

This case report supports the vigorous application of measures to increase adherence to protocols after biological contamination. We believe that all cases, if there are no contraindications, should be evaluated promptly to prescribe a standard full antiviral schedule of pegylated interferon and weight-based ribavirin, especially in cases like this one in which there is a viral kinetic showing a transient or slow resolution of the active infection. Currently, response-guided therapy helps to determine the duration of treatment, which is cost saving and more advantageous for patients[40]. In this case report, the RVR allowed for the use of a shorter schedule, 24 wk of therapy, which is approximately 12.000 € less than the standard of 48 wk.

In conclusion, the lessons from this case are as follows: (1) health care workers must be counselled and trained to avoid occupational exposure; (2) all injured cases must report the exposure immediately and undergo standard procedures; and (3) a weight-adjusted full schedule of the standard regimen with pegylated interferon and ribavirin should be proposed in all cases with acute HCV infection showing an active and longer viral replication, in particular for cases with more than 6 mo of acquisition. These suggestions might help to avoid the unaware HCV-carrier status, and thus some chronic infections with the potential progression to cirrhosis and to major complications, such as hepatocellular carcinoma.

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CASE REPORT

Duplication of the transverse colon in an adult: Case report and review

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Abstract

Tubular duplication of the colon is very rare especially in adulthood, because it is frequently symptomatic earlier in newborn life, so only few cases are reported in literature. Several theories are proposed to explain the onset and the evolution of gut malformations as the aberrant lumen recanalization or the diverticular theory, the alteration of the lateral closure of the embryonal disk or finally the dorsal protrusion of the yolk-sac for herniation or adhesion to the ectoderm for an abnormality of the longitudinal line, but none clarifies the exact genesis of duplication. We present a case of "Y-shaped" tubular duplication of the transverse colon in a 21-year-old adult, with a history of chronic pain and constipation, referred to our department for abdominal pain with retrosternal irradiation, treated with the resection of the aberrant bowel.

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Key words: Colonic diseases; Colorectal duplication; Neogenesis; Colonic duplication; Transverse duplication

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INTRODUCTION

Duplications of gastrointestinal tract are infrequent in general population with an incidence of two or three cases per year in referred pediatric center[1]. The most frequent localization is the ileum 30% and the ileocecal valve 30%, followed by the jejunum with 8%, the colon 6%-7% and the rectum 5%[2,3]. Since 1950, less than 50 colonic duplication have been reported in literature[4] and they were more often discovered in the first two years of life (80%)[5]. If no associated malformation is present, they remain frequently hidden for several years until a complication occurs[6]. The symptoms are unspecific and depend on the type of duplication and on the associated abnormality. More often they are abdominal mass, chronic pain, constipation, occlusion, and less frequently volvulus[7] intussusception, bleeding[8] or perforation (especially in sigmoid colon mimicking diverticulitis). Also the arising of carcinoma is observed: the most frequent is adenocarcinoma[9-13] followed by squamous carcinoma and carcinoid tumors[14-16]. The carcinoembryonic antigen expression can be observed in association with the arising of adenocarcinoma[17], but the presence of the carbohydrate antigen 19-9 is not well defined yet.

CASE REPORT

A 21-year-old man with a history of chronic constipation was referred to our department for abdominal pain with retrosternal irradiation. During childhood he was hospitalized for the same symptoms without a clear diagnosis:

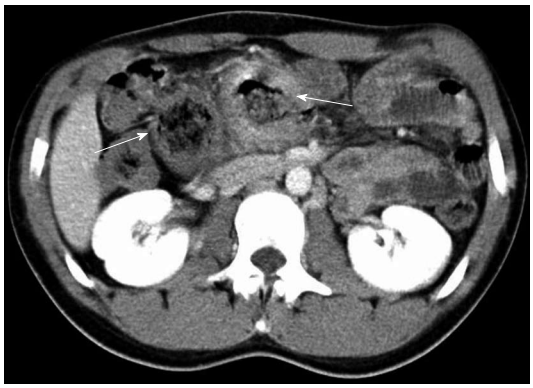

Figure 1 Computed tomography scan. The arrows indicate the duplication.

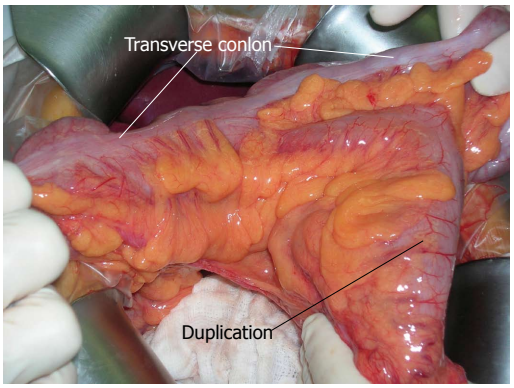

Figure 2 Intraoperative dissection.

alimentary intolerance or celiac disease were excluded, and all the abdominal ultrasounds were always negative. The mother had no particular problems during pregnancy and labour, she had no exposition to pollution, but the patient was an eight months premature birth. At the hospitalization the blood samples showed mild leukocytosis and the abdominal Rx plane was normal. Abdominal sonography was performed but it was not diagnostic, because of air hiding; an abdominal computed tomography (CT) scan (Figure 1) revealed an oval mass with wall enhancement and similar stool material inside, suggestive for intestinal duplication. A small-bowel contrast study was performed but no alteration could be demonstrated. The patient was treated with antibiotics and was discharged asymptomatic with a planned intervention for the following week. At the explorative laparotomy a transverse colon Y-type duplication was shown (Figure 2); the duplication was bent down at the origin of the transverse mesocolon and an own mesocolon was seen. After dissection from the mesocolic stamp, the duplication was unbent revealing that the blood supply origin from a distal division of the middle colic artery with a trifurcation for right and left transverse colon and one branch for the duplication. The upper part of the common mesocolon presented a crabbed fusion of small vessels. To preserve the left marginal arcade, we dissected the artery for the duplication with conservation of the two branches for the native transverse colon and with resection of the duplicated bowel and 5 cm of the native transverse colon. A colo-colic anastomosis was performed. The post operative period was uneventful and the patient was discharged; at the moment he lies symptoms free. Histopathology confirmed the tubular duplication with multiple mucosal ulceration.

DISCUSSION

Digestive duplication is defined by the three Rowling's criteria[18]: (1) the wall of the duplication is in continuity with one of the duplicated organ; (2) the cyst is surrounded by a smooth muscular layer; and (3) a layer of digestive mucosa is present, more often typical or heterotopic as gastric mucosa, colonic mucosa, bronchial or pancreatic structure[19,20]. Smith[21] well defined the term of duplication as "these formations, either tubular or spherical, which lie in contiguity with normal bowel and which share with it a common blood supply and usually a common muscle coat. There may or may not be a communication between the normal and anomalous lumina and the dividing septum may be muscular or merely a double layer of epithelium". Gross *et al*[22] described four variations in the shape of duplications: (1) a tubular structure that branches out from the intestine and extends for some distance between the mesenteric leaves; (2) a double-barreled structure communicating with the intestinal lumen at one or both ends; (3) a cystic structure lying in the peritoneal cavity, attached by a mesenteric stalk; and (4) a spherical lesion contiguous with some part of the bowel, particularly along the ileum. Cystic types are the most frequent with 90%-95% of the cases and the tubular type 5%-10%; the latter can be distinguished in double barreled (80%) or Y-shaped form (20%)[23-26]. For colonic duplication McPherson makes another classification as type I (simple cysts), type II (diverticula) or the most common type III (tubular colonic duplication)[27]. Several theories have been proposed to describe this abnormality in which environmental factors, such as trauma or hypoxia, can play a role[28]. The aberrant lumen recanalization theory exposed by Bremer[29] seems to be the most comprehensive. As described by Johnson[30] in 1910 the gut grows and obliterates the lumen at the sixth week with subsequent internal vacuolization and coalescence of vacuoles with the formation of the single lumen. The aberrant vacuolization well explains that vacuoles can remain separated and create one or more duplication that can be separated or communicating with the original gut. This theory can be easily associated to the double barrel duplication. Another theory is the diverticular one, described by Lewis *et al*[31], criticized by some authors[1] because it doesn't explain the forms of entire duplication of the colon (for example) with the presence of circular and longitudinal muscle coats. Blinder describes more simply that the tubular forms can be caused by an alteration of the lateral closure of the embryonal disk at the stage 9 of Steeter for an abnormality of the longitudinal line, instead of

cystic forms that originate from diverticulation later in the evolution[32,33]. Another explanation, well described by Smith[21], is the dorsal protrusion of the yolk-sac caused by its herniation or adhesion to the ectoderm in same phase of presomite development taking to a "dorsal remnants" structure, that can be divided into: congenital dorsal enteric fistula, congenital dorsal enteric sinus, congenital dorsal enteric cyst, congenital dorsal enteric divericulum[34]. This last theory can also explain the association with other abnormalities, especially the vertebral alteration. The association of other malformations, as vertebral or genitourinary alterations, can be classified in the notochordodysraphies: duplication or alteration of the hindgut is associated with genitourinary problem in 60% of cases[35,36] and high malformation with vertebral problems[37-40]. In particular the hindgut duplication can be explained by an insult to the caudal cell mass at the days 23-25 resulting in the "split notochord syndrome[41,42]. Until 2011 only 18 cases of transverse colon duplication have been reported. The particular localisation sometimes can give problems in diagnosis, like mismatching with pancreatic cyst[5,43]. In tubular type, CT scan, contrast enema and colonoscopy, could be the best diagnostic tools: in fact the presence of air and stool can facilitate the diagnosis; in ultrasonography the presence of air doesn't actually allow a good vision. Probably sonography, instead of CT scan, can be more performant in the cystic type, where the diagnosis is more difficult as described above. In fact in the cystic type of the transverse colon, the lesion have fluid inside and it could be adherent to the mesocolon or the pancreas, mimicking other alterations. The small-bowel contrast study, as in our case, is not so performant also in tubular type. The management of colonic duplication consists in the resection of the duplicated bowel with an extension of 2 cm in the normal bowel, due to the common blood supply at the two organs, the presence of fibrosis in the conjunction area[6,44] and the probability of arising tumor in the duplication. Our report shows that the symptomatology could be hidden for a long time, especially in absence of other anatomical alterations; probably for the presence of a big communication with the native bowel. The presence of stool inside the duplication helped us in the diagnosis, but it remains not easy to do, as reported by some authors[43]. In conclusion the colon duplication is not frequent especially in adulthood; the differential diagnosis is not easy; it must be resected even if asymptomatic, because of the risk of arising tumor.

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CASE REPORT

Steroid-refractory ulcerative colitis and associated primary sclerosing cholangitis treated with infliximab

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Abstract

Primary sclerosing cholangitis is an infrequent extraintestinal manifestation of ulcerative colitis. Damage to bile ducts is irreversible and medical therapies to prevent progression of the disease are usually ineffective. We describe a patient with long-standing ulcerative colitis, which was refractory to corticosteroid therapy who developed primary sclerosing cholangitis (biochemical stage Ⅱ/Ⅳ) in the course of his pancolitis. Treatment with infliximab (5 mg/kg as an induction dose followed by maintenance doses every two months) was indicated because of steroid-dependent disease associated to primary sclerosing cholangitis as well as sacroiliitis and uveitis and previous episode of severe azathioprine-related hepatic toxicity. At present, after two years of follow-up, the patient is asymptomatic with normal liver tests and complete resumption of daily life activities. This case draws attention to the usefulness of anti-tumor necrosis factor-alpha therapy for the management of primary sclerosing cholangitis as extraintestinal manifestation of inflammatory bowel disease.

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Key words: Ulcerative colitis; Infliximab; Monoclonal antibodies; Sclerosing cholangitis; Bile duct diseases; Tumor necrosis factor-alpha

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INTRODUCTION

Inflammatory bowel diseases are associated with extraintestinal manifestations involving almost every organ system in the body, including the musculoskeletal, dermatologic, hepatic, pancreatic, biliary, ocular, renal and pulmonary systems and can cause a significant challenge to physicians managing patients with Crohn's disease and ulcerative colitis[1-3]. Primary sclerosing cholangitis, a chronic, progressive disorder of unknown etiology that manifests as inflammation, stricturing, and fibrosis of medium and large intra- and extrahepatic bile ducts, is one of the most serious complications of inflammatory bowel disease, with an established strong relationship with ulcerative colitis[4]. At least 75% of patients with primary sclerosing

cholangitis have coexisting ulcerative colitis[2]. However, only 5% of patients with ulcerative colitis develop primary sclerosing cholangitis. The clinical course of sclerosing cholangitis bears no relationship with the underlying inflammatory bowel disease but damage to bile ducts is irreversible, no medical therapies have been shown to be effective at preventing the progression of the disease, and orthotopic liver transplantation is the only curative treatment.

The advent of biologic response modifiers, e.g., tumor necrosis factor-alpha (TNF-α) inhibitors, has improved the treatment of inflammatory bowel disease and its associated extraintestinal manifestations[5], such as arthritis and uveitis[6,7]. Current data suggest that infliximab is an effective alternative treatment option for patients with moderate to severe ulcerative colitis with an inadequate response to conventional glucocorticoid treatment[8]. However, the efficacy of infliximab in primary sclerosing cholangitis in patients with ulcerative colitis has not been previously assessed.

We report the case of a patient with steroid-refractory ulcerative colitis with intolerance to thiopurines and various extraintestinal manifestations, including ankylosing spondylitis and primary sclerosing cholangitis with favorable response to infliximab therapy.

CASE REPORT

We report here on a 68-year-old man in whom positive HLA B27 spondyloarthropathy and uveitis in the left eye were diagnosed at the age of 54. He was referred to the hospital because of an episode of subacute diarrhea with blood and mucous associated with mild iron-deficiency anemia. Also, he was diagnosed endoscopically and histologically of mild to moderate ulcerative rectosigmoiditis. He received oral and topical 5-aminosalicylic acid with good initial response. During the course of the disease, he presented multiple episodes of steroid-dependent ulcerative colitis and treatment with azathioprine was started 6 years after diagnosis. After 4 mo, azathioprine was discontinued due to severe hepatic cytolysis. Four years later, he was readmitted to the hospital because of bloody diarrhea and mucus (12-14 bowel movements daily, with generalized abdominal pain, proctalgia, fecal urgency and tenesmus without fever, and weight loss of 6 kg. Physical examination showed marked impairment of the patient's general condition, diffuse abdominal pain on palpation and functional limitation secondary to ankylosing spondylitis. Blood tests showed serum hemoglobin 10.5 g/dL, C-reactive protein 154 mg/dL and cholestasis hepatitis with bilirubin 1.2 mg/dL, aspartate aminotransferase (AST) 112 IU/L, alanine aminotransferase (ALT) 162 IU/L, alkaline phosphatase 281 IU/L, gamma-glutamyl transpeptidase (γ-GGT) 913 IU/L, and positive p-antinuclear antibodies 1/80. A diagnosis of moderate to severe episode of pancolitis was established. A cholangio-magnetic resonance imaging (MRI) study disclosed stenosis of the proximal common bile duct probably related to cholangitis. Causes of secondary sclerosing cholangitis were excluded as shown by the lack of abnormally elevated immunoglobulin G4 (IgG4) levels as shown in autoimmune pancreatitis or IgG4-related sclerosing cholangitis (IgG4 levels were < 100 mg/dL). Also, the patient showed increased values serum bilirubin and alkaline phosphatase of a lower magnitude that those suggestive of autoimmune pancreatitis, and radioimaging findings for enlargement of the pancreatic gland were absent. Also, the patient did not complain of abdominal symptoms suggestive of pancreatitis. All these data together with the presence of ulcerative pancolitis directed us to confirm the diagnosis of primary sclerosing cholangitis associated to inflammatory bowel disease and to exclude the diagnosis of autoimmune pancreatitis.

The patient was diagnosed of primary sclerosing cholangitis (biochemical stage Ⅱ/Ⅳ). He was treated with full doses of *i.v.* corticosteroids and urodesoxycholic acid 15 mg/kg body weight, without improvement. The patient received full doses of methylprednisolone, 1 mg/kg per day, with subsequent dose reductions at least on seven occasions over the course of 6 years. The situation of the patient was re-assessed and decided to start treatment with infliximab (5 mg/kg as an induction dose followed by maintenance doses every two months) because of steroid-dependent disease associated to primary sclerosing cholangitis, sacroiliitis and uveitis and previous history of an episode of severe azathioprine-related hepatic toxicity. At present, after two years of follow-up, the patient is still on treatment with infliximab and has remained asymptomatic, with improvement of anemia (hemoglobin 13.8 mg/dL) and biochemical evidence of cholestasis (bilirubin 0.4 mg/dL, AST 16 IU/L, ALT 20 IU/L, alkaline phosphatase 59 IU/L, γ-GGT 149 IU/L) and complete restoration of the quality of life. Annual endoscopic assessment did not show signs of dysplasia. Repeated cholangio-MRI performed during the follow-up was also unrevealing.

DISCUSSION

The incidence of primary sclerosing cholangitis varies between 0.9 to 1.6 per 100 000 persons/year[9]. More than two-thirds of patients are males and the most commonly associated condition is an inflammatory bowel disease which occurs in up to 70% of affected subjects. Inflammatory bowel disease in primary sclerosing cholangitis patients represents a distinct phenotype in that pancolitis is observed in 94% of patients with ulcerative colitis and in 96% of patients with Chron's disease[10]. It has been shown large differences between primary sclerosing cholangitis patients with and without concurrent inflammatory bowel disease. Patients with inflammatory bowel disease showed earlier appearance of primary sclerosing cholangitis than those without inflammatory bowel disease and are more likely to develop serious malignant complications and more likely to require liver transplantation[11].

In our patient, distal colitis was the initial manifestation of inflammatory bowel disease and later evolving to pancolitis, the time at which primary sclerosing cholangitis developed. Primary sclerosing cholangitis has been shown to be associated with greater anatomic extent of colitis[12-14]. It has been reported that development of primary sclerosing cholangitis in patients with ulcerative colitis may have a positive effect on colonic disease[15,16] with reduced disease activity and less use of steroids, azathioprine and surgery[17]. In other studies, ulcerative colitis associated with primary sclerosing cholangitis showed unique colonoscopic features (pancolitis, rectal sparing and backwash ileitis) with more frequent colorectal neoplasia development and worse prognosis than ulcerative colitis patients without primary sclerosing cholangitis[18]. In our patient the clinical course was characterized by severe ulcerative colitis refractory to steroid therapy. For this reason and taking into account the presence of other concomitant manifestations (uveitis, sacroiliitis) treatment with infliximab was started. However, indications of anti-TNF-α in well established primary sclerosing cholangitis should be carefully balanced due to immunosuppression and the risk of potentially fatal cholangitis.

The use of infliximab was followed by marked improvement in the patient's clinical condition, including the extraintestinal manifestations of ulcerative colitis and a favorable clinical and biochemical remission of primary sclerosing cholangitis. This suggests a direct effect of infliximab on hepatic inflammation. However, in a double-blind, placebo-controlled study of 24 patients with primary sclerosing cholangitis, no significant treatment benefit of infliximab was demonstrated[19], although in patients with primary sclerosing cholangitis and Crohn's disease treatment with infliximab was associated with improvement of liver function tests[20].

Currently, the use of anti-TNF-α treatment in primary sclerosing cholangitis is not well established. The observation that reduced T cell reactivity in liver infiltrating cells obtained from patients with primary sclerosing cholangitis was due to high local production of TNF-α provides support for the use of anti-TNF antibodies as an alternative treatment for these patients[21]. Contrarily, in the experience of Epstein *et al*[22] etanercept was well tolerated but not effective in a clinical series of 10 patients with clinically active primary sclerosing cholangitis. It is unknown whether early treatment with anti-TNF-α drugs may change the natural history of primary sclerosing cholangitis. Also, the use of this medication over years may raise financial concerns and secondary effects of this prolonged use. In the case here presented, although treatment with infliximab is expensive, the patient did not present recurrent episodes of colitis, uveitis, sacroiliitis or new episodes of cholangitis, allowing prompt resumption of work and social activities with an excellent quality of life and without further admissions to the hospital or the need of surgical operations, as a result of which direct and indirect costs have been markedly reduced in this particular case.

In conclusion, our report on a patient with steroid-refractory ulcerative colitis developing primary sclerosing cholangitis with favorable and maintained response to infliximab therapy draws attention to the usefulness of anti-TNF-α therapy for management of primary sclerosing cholangitis as extraintestinal manifestation of inflammatory bowel disease.

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CASE REPORT

Iatrogenic esophago-tracheal fistula: Challenges in diagnosis and management

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Abstract

Esophageo-tracheal fistula is a rare condition, and in most cases such fistulas are caused by malignant disease or emergency endotracheal intubation. A case where a wrapped tablet produced a fistula between the esophagus and trachea is described. The patient is a male born in 1938 who swallowed a tablet without unwrapping it. The patient was treated with self-expanding metal stents (SEMS), but closure of the fistula was not achieved. Different examinations and treatment options are discussed. Surgical treatment for this condition has demonstrated considerable mortality and morbidity. In some cases closure of the fistula can be achieved by use of SEMS. Although we advise treatment of such cases with SEMS, in some cases treatment with stents will prove troublesome and the risk/benefit analysis will have to be reevaluated.

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Key words: Esophageo-tracheal fistula; Stenting; Iatrogenic; Percutaneous endoscopic gastrotomy; Surgery

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INTRODUCTION

Esophagotracheal fistula is a rare condition, and in most cases such fistulas are caused by malignant disease or emergency endotracheal intubation. We describe a case with a unique etiology where a wrapped tablet produced a fistula between the esophagus and trachea. Challenges in diagnosis and treatment are described.

CASE REPORT

The patient is a male born in 1938. In 1997 he had prosthetic hip surgery, for which he was re-operated in 2008. Parkinsons disease was diagnosed in 2009, and he suffers from benign prostatic hyperplasia.

In 2008, during hospitalization for the hip-reoperation, he was given four tablets by a staff nurse as ordinated by a physician. Upon swallowing the tablets, the patient felt intense chest discomfort, and it was speculated whether he had swallowed one of the tablets without unwrapping it. No further investigation was done at this point.

In 2010 the patient was referred to the Department

Figure 1 Foreign body measured 2 cm x 2 cm and the tablet remained intact inside the wrapping.

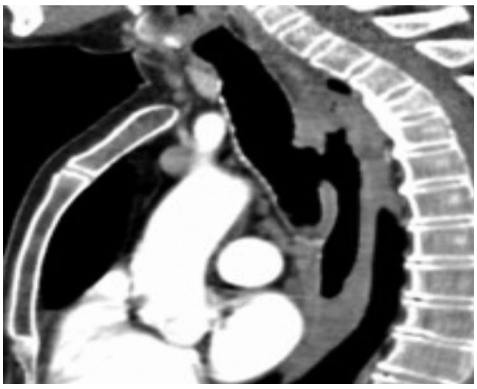

Figure 2 Computed tomography confirmed a fistula, 20 mm x 6-7 mm, at aortic arch level.

of Gastroenterology because of dysphagia.

An upper endoscopy was performed and at 25 cm from the teeth the endoscopist discovered a stenosing process with mucosal "irritation" and signs of ulceration. Biopsies from the area revealed ulceration, but no signs of malignancy. Distally, the esophagus was normal. He had signs of gastritis with a positive *Helicobacter pylori*-test. The duodenum was normal.

The initial computer tomography (CT) raised suspicion of malignancy, as it described a 4-5 cm tumor of the esophageal wall and adjacent pathological lymph nodes in the mediastinum.

Three weeks later a new endoscopy was performed, and the suspicion of a foreign body was raised. The structure in the esophagus had a blueish discoloration, and the consistency was "hard as a rock".

The patient was referred to a tertiary centre for possible endoscopic removal of a foreign body but, due to the risk for perforation, instead of trying to remove the foreign body a self-expanding, fully covered esophageal stent was placed. The patient now was able to eat.

Five months later the patient came back after having coughed up a foreign body. He brought the item to the outpatient clinic. Upon inspection it measured 2 cm × 2 cm and the tablet remained intact inside the wrapping (Figure 1). The patient reported chest discomfort, but no respiratory symptoms. Another endoscopy was performed. The stent was in place, and there was no visible fistula. Biopsies showed inflammation and proton pump inhibitor treatment was initiated.

Again a CT was performed, describing fibrotic changes in the right lung, and low-grade brochiectasia. No contrast leakage between the esophagus and the respiratory tract was seen.

The patient was in good health, but felt some discomfort having a stented esophagus. An informed decision was made to attempt stent removal under anaesthesia.

The stent was removed endoscopically, and 15 min after the procedure bleeding from the endotracheal tube was noticed. There was no subcutaneous emphysema. The patient was extubated and was able to breathe normally. The suspicion of a esophago-tracheal fistula was raised and antibiotic therapy was initiated. CT confirmed a fistula, 20 mm × 6-7 mm, at aortic arch level (Figure 2).

In order to - as far as possible - prevent aspiration from the esophagus to trachea, and to see if a spontanous closure of the fistula was achievable, a percutaneous endoscopic gastrostomy (PEG) was placed. This also ensured nutritional adequacy.

The patient was observed as an outpatient, and attended two control CTs. The fistula persisted unchanged.

After another three months it was decided to place a new esophageal stent. Upon esophagoscopy the fistula still measured 20 mm × 7 mm, and there was direct visability into the carina and the left and right main bronchus. The PEG was removed, and a fully covered, self-expanding stent was placed to cover the fistula. We chose a stent with a diameter of 28 mm at the two ends in an attempt to avoid food/liquids from getting access from the esophagus to the trachea. The length of the stent was 12.5 cm and the diameter in the center of the stent was 23 mm. A bronchoscopy was performed, during which the stent was directly visible from the tracheal side (Figure 3). A CT was performed, showing the stent in place (Figure 4).

Two months after the last stent placement the patient is asymptomatic, and is obtaining natural nutrition. A follow-up CT is scheduled.

DISCUSSION

A report on surgical correction in two cases of iatrogenic esophago-tracheal fistulas is available[1]. These fistulas were secondary to traumatic emergency endotracheal intubation. Another report on four patients with iatrogenic fistulas demonstrated considerable mortality and morbidity in relation to emergent surgery[2]. Two patients were under long term treatment in the ICU, one suffered a stroke, and one died. A study on four patients with esophago-tracheal fistula being treated with self-expanding oesophageal stents reported fistula closure in two patients[3]. A review on surgical techniques is available[4], outlining the invasiveness of the procedures. Management of an iatrogenic tracheo-esophageal fistula

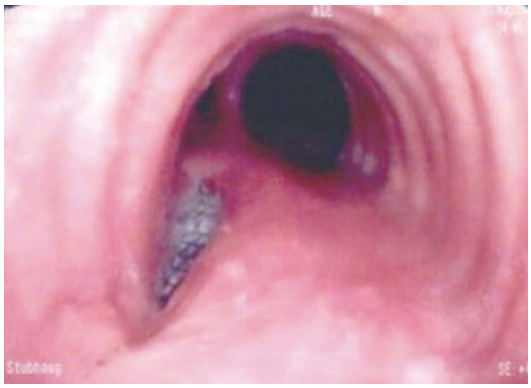

Figure 3 Bronchoscopy was performed, during which the stent was directly visible from the tracheal side.

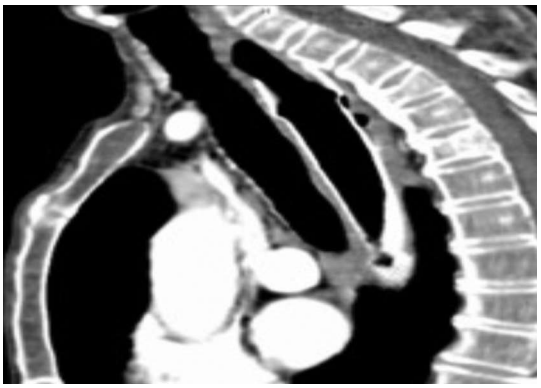

Figure 4 Computed tomography was performed, showing the stent in place.

using a Y-stent on the tracheal side is described[5]. A self-expanding metal stent is, according to one study, safe and effective in palliation of malignant fistulae in selected patients[6]. However, this study was conducted on 90 patients with malignant changes in the esophagus, of which only 5 patients had a fistula.

Our case illustrates a unique etiology of an esophago-tracheal fistula. The patient presents a continuous challenge in treatment. Discussions and considerations with thoracic surgeons have so far concluded with stenting as the treatment of choice. The patient's age and comorbidity is being taken into account in this decision. However, if the management with esophageal stents proves troublesome, the risk/benefit analysis will have to be re-evaluated.

The wrapped tablet was forced from the esophageal wall to the trachea, probably powered by the self-expanding stent. Long-term stenting has so far given no reduction in the size of the defect.

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CASE REPORT

Fulminant gastrointestinal graft-versus-host disease concomitant with cytomegalovirus infection: Case report and literature review

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Abstract

Here, we report a case of fulminant gastrointestinal graft-versus-host disease (GI-GVHD) with cytomegalovirus (CMV) infection in 44-year-old woman. Despite the difficulties associated with the treatment of GI-GVHD and GI-CMV disease, the mucosal findings and the clinical course showed marked improvements during long-term clinical observation. The endoscopic findings were remarkable, with diffuse sloughing mucosa in the stomach and highly active inflammation and deep discrete ulcers throughout the colon. Changes in the CMV quantitative polymerase chain reaction results were correlated with the endoscopic mucosal findings and were useful for assessing the efficacy of the treatment. Although a definite diagnosis of GI-GVHD is generally made by endoscopy with biopsy, the gross appearance of this disease can vary depending on the endoscopy. In this paper, we also conduct a literature review of patients with GI-GVHD.

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Key words: Acute gastrointestinal graft-versus-host disease; Allogenic stem-cell transplantation; Cytomegalovirus gastrointestinal disease; Cytomegalovirus-polymerase chain reaction; Endoscopy

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INTRODUCTION

Graft-versus-host disease (GVHD) is a serious complication of allogeneic hematopoietic stem cell transplantation (HSCT) and mainly attacks the skin, gastrointestinal (GI) tract and liver[1-5]. GI-GVHD, in particular, can cause life-threatening complications, such as massive diarrhea, hemorrhage, paralytic ileus, and perforation[6,7]; therefore, the accurate diagnosis of GI-GVHD is essential. Although endoscopic observation is an indispensable diagnostic tool, various mucosal patterns are observed on endoscopy[8]. In addition, testing for cytomegalovirus (CMV) infection is necessary because the complication of GI-GVHD with CMV infection will reduce the quality of life (QoL) of the patients[9]. This reduction in the QoL occurs because CMV-related GI disease is exacerbated by the administration of steroids for the treatment of GI-GVHD when antiviral drugs are not administered[10]. Here, we report a rare case of fulminant GI-GVHD detected by endoscopy and its clinical course, and we review related literature on the endoscopic findings of GI-GVHD.

CASE REPORT

The patient, a 44-year-old woman with acute myeloid leukemia who was diagnosed 18 mo earlier, underwent

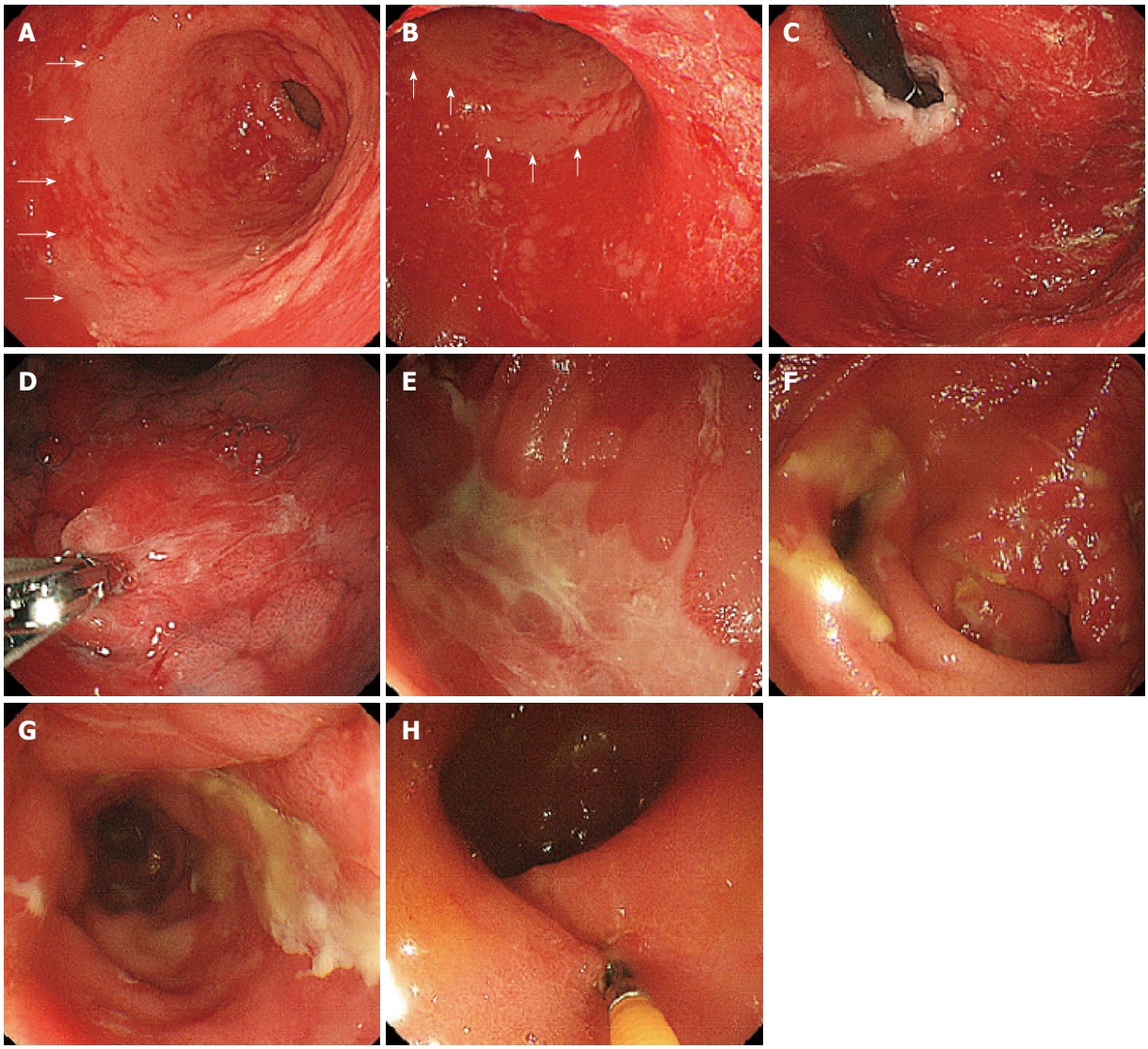

Figure 1 Upper and lower gastrointestinal endoscopic findings. A: The gastric mucosa in the antrum of the stomach. The antrum is the only segment exhibiting normal mucosa (arrows); B: The gastric mucosa in the lower segments of the corpus of the stomach. This mucosa demonstrates diffuse sloughing, whereas the antrum is the only segment exhibiting normal mucosa (arrows); C: The gastric mucosa in the upper segments of the corpus of the stomach; D: The greater curvature of the stomach near the center. A biopsy was taken from the edematous and sloughing mucosa of the greater curvature of the upper corpus; E: Shallow ulcer with fur at the cecal valve and cecum; F: Discrete longitudinal ulcer in the terminal ileum; G: Longitudinal ulcer with fur surrounded by the inflamed edematous mucosa of the sigmoid colon; H: Edematous mucosa of the rectum.

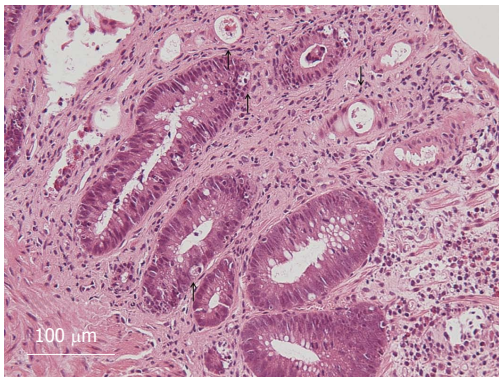

Figure 2 Histological findings. Biopsy specimen from the sigmoid colon revealing a crypt with multiple apoptotic cells (arrows) with severe lymphocytes corresponding to acute gastrointestinal graft-versus-host disease (GVHD). The presence of gland apoptosis is histological evidence of acute GVHD (hematoxylin and eosin stain, × 40).

allogeneic bone marrow transplantation 3 mo prior to the examination. A full-body skin rash appeared on day 26 after the transplantation. On day 85 after the transplantation, she experienced watery diarrhea more than 10 times per day and developed abdominal pain. Upper GI endoscopy showed diffuse sloughing of the mucosa with diffuse erythema and hemorrhage in the antrum and corpus of the stomach (Figure 1A-D). Lower GI endoscopy revealed multiple deep discrete ulcers with exudative and mucosal oozing in the terminal ileum end and throughout the entire colon (Figure 1E and F). Biopsy specimens from the upper and lower GI tract revealed diffuse erythema, erosions, and sloughing mucosa with active bleeding in the stomach, as well as multiple erosions and a small discrete ulcer with active bleeding in the lower GI tract. Gland apoptosis showed histological evidence of acute GVHD (Figure 2). Moreover, the

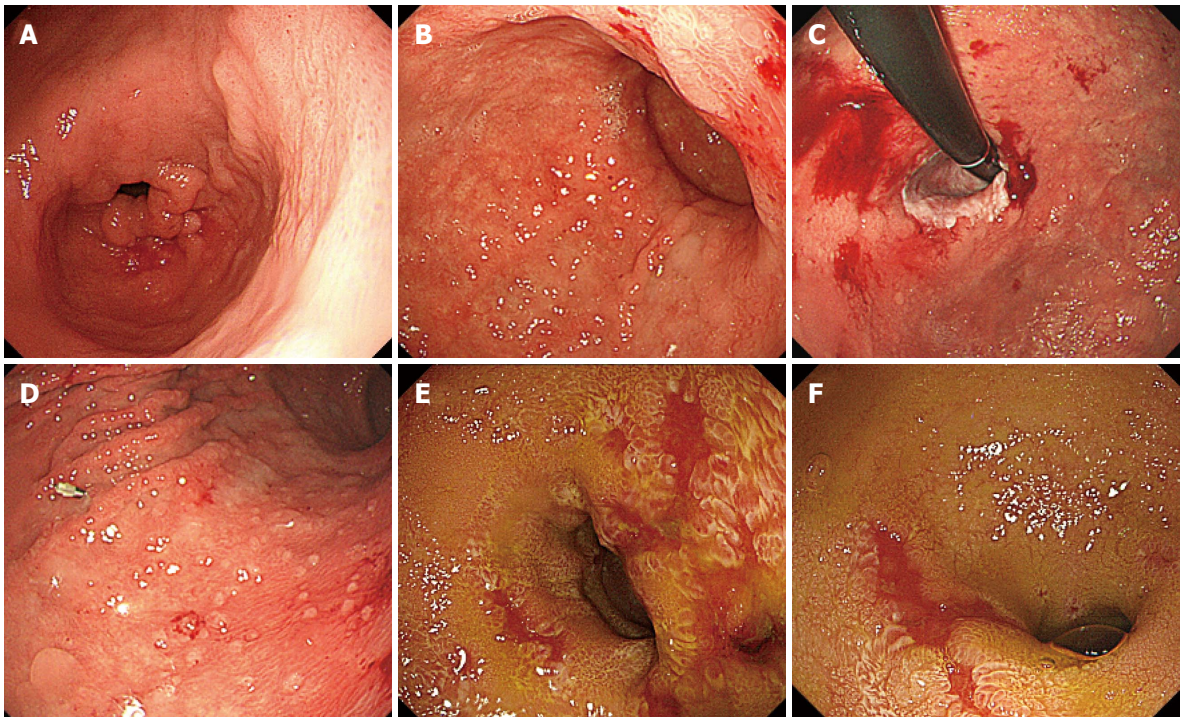

Figure 3 Upper and lower gastrointestinal endoscopic findings 70 d after the steroid therapy. A: The gastric mucosa in the antrum of the stomach. The mucosa is normal but somewhat edematous; B: The gastric mucosa in the lower segments of the corpus of the stomach, which has improved even though the rough gastric mucosal patterns and edematous changes remained; C: The gastric mucosa in the upper segments of the corpus of the stomach, which has also improved; D: The greater curvature of the stomach near the center. The endoscopic clipping has remained; E: Sigmoidoscopy revealing multiple discrete ulcers in the sigmoid colon; F: Disappearance of the visible vascular pattern in the sigmoid colon.

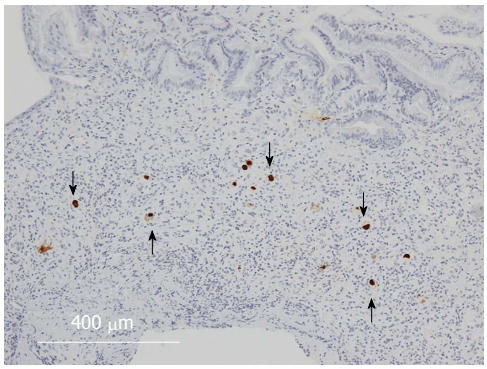

Figure 4 Immunostaining of the biopsy specimen. Immunohistochemically stained biopsy specimen from an ulcer of the sigmoid colon showing multiple cytomegalovirus-positive cells (arrows) (immunohistochemical stain, × 40).

biopsy specimens showed CMV infection detected by polymerase chain reaction (PCR) at remarkably elevated concentrations of 4.0×10^4 copies/μg DNA, but immunochemical staining for the CMV antibody was negative. Finally, the patient was diagnosed with severe GI-GVHD concomitant with a CMV infection. Other organ involvement included a whole-body skin rash and slight liver dysfunction.

The treatment for acute GVHD was started with an initial dose of prednisolone 1 mg/kg per day (50 mg/d) and for CMV infection with an initial dose of ganciclovir (GCV) 10 mg/kg per day. After 40 d of treatment, the patient improved clinically. Then, GCV was discontinued because of thrombocytopenia that appeared 60 d after the treatment. After 64 d, however, CMV was detected in the blood by a CMV-antigenemia assay, and the maintenance dose of GCV (5 mg/kg per day) was resumed. After 70 d, upper and lower endoscopy showed that the endoscopic findings of mucosal sloughing had improved (Figure 3A-D) but that the colonic mucosa remained ulcerative and inflamed (Figure 3E and F). Both upper and lower GI tract biopsies revealed CMV-infected cells in immunohistochemically stained tissue specimens (Figure 4), and the CMV-PCR from the GI tract biopsy was 2.0×10^5 copies/μg DNA. Intravenous GCV at 5 mg/kg every 12 h as the induction therapy was re-started and then changed to the maintenance therapy, and the prednisolone dose was tapered. Although the treatment continued for an additional 102 d, the CMV-PCR from the GI tract biopsy was 1.0×10^3 copies/μg DNA. Oral valganciclovir at 900 mg/d was started, with oral prednisolone 10 mg administered every other day. After 162 d, the upper and lower endoscopy showed notable improvements in the mucosal findings (Figure 5), and the CMV-PCR count from a GI tract biopsy was within the normal range of $< 4.0 \times 10$ copies/μg DNA.

DISCUSSION

Nausea and vomiting, appetite loss, abdominal pain, and watery diarrhea are common symptoms of GI-GVHD. Watery diarrhea appears in almost all cases of GI-GVHD and occasionally becomes chronic or causes bleeding. Therefore, this symptom is a major factor that reduces

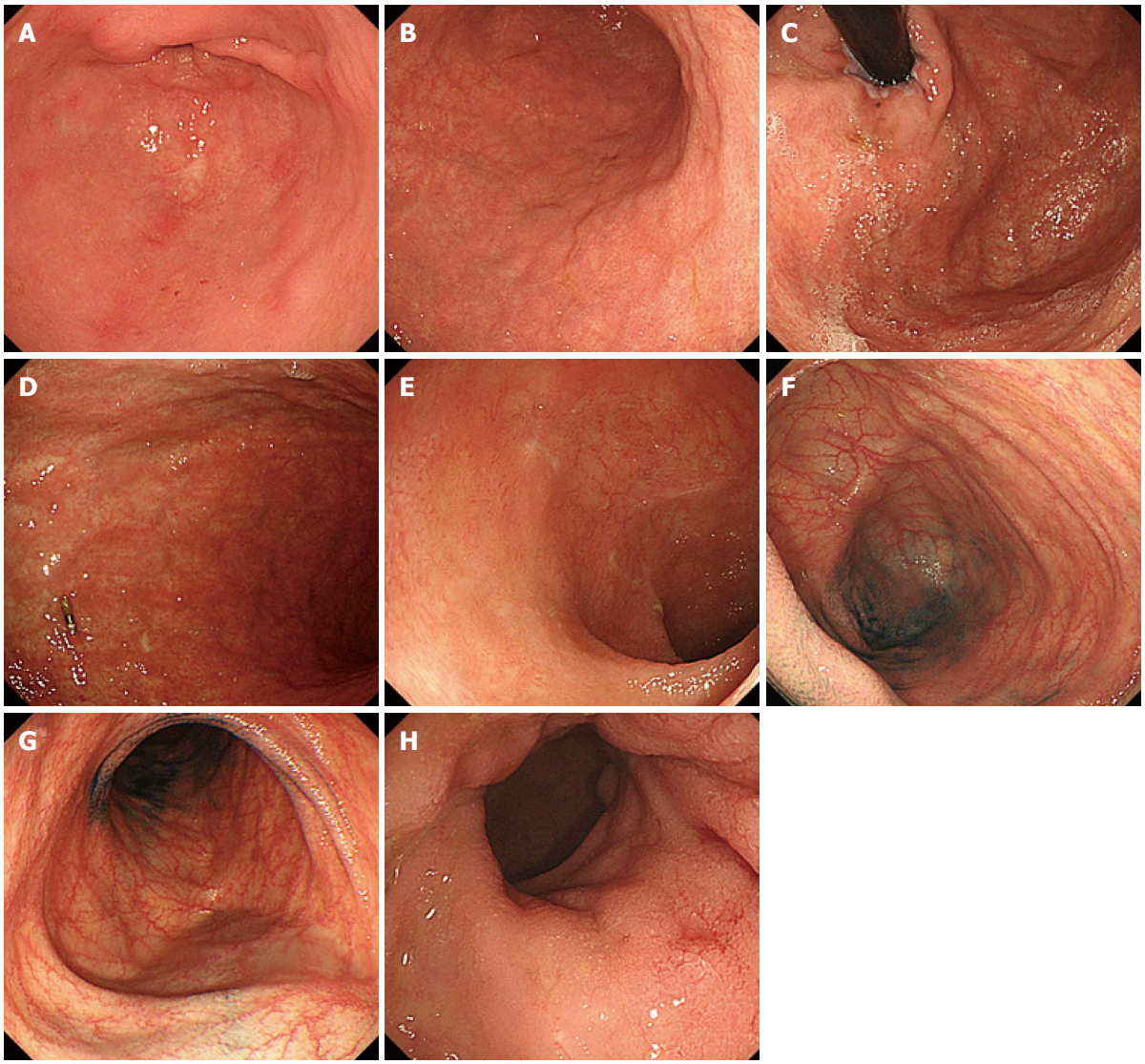

Figure 5 Upper and lower gastrointestinal endoscopic findings 162 d after the steroid and anti-cytomegalovirus therapies. A: The gastric mucosa in the antrum of the stomach; B: The gastric mucosa in the lower segments of the corpus of the stomach; C: The gastric mucosa in the upper segments of the corpus of the stomach; D: The greater curvature of the stomach near the center. The endoscopic clipping has remained, and nearly the entire mucosa of the stomach has improved to a normal state; E: Normal mucosa in the cecal valve and cecum; F: Normal mucosa in the terminal ileum; G: Marked improvement of the inflammation of the colonic mucosa, and healed ulcers in the sigmoid colon; H: The vascular patterns are now visible, indicating that the normal mucosa has returned in the rectum.

patient QoL[11]. Moreover, after HSCT, CMV infection of the GI mucosa and GI-GVHD can both cause diarrhea[12,13].

Diseases characterized by the development of GI symptoms after HSCT include not only CMV and GI-GVHD infection but also virus infections caused by enterovirus, adenovirus, rotavirus, and Epstein-Barr virus, complications with bacterial and fungus infections, and colitis associated with thrombotic microangiopathy and regimen-related toxicity. In routine clinical examination, differentiating among these diseases is difficult[14], particularly because GI-GVHD is frequently associated with CMV infection[15,16]. In this case, because of the severe GI-GVHD accompanied by CMV infection, the patient developed frequent and long-term bloody diarrhea and abdominal pain, which appeared to have led to serious symptoms such as anemia, malnutrition, and dehydration.

The endoscopic findings reflected the severity of the clinical symptoms. The lower GI tract exhibited severe signs of multiple deep ulcers, edema, and erythema, with no normal mucosa observed. The upper GI endoscopy revealed diffuse sloughing of the mucosa in the stomach, except for the antrum. Although the endoscopic findings of chronic GI-GVHD may include the characteristic esophageal web and strictures[2], various endoscopic findings are associated with acute GI-GVHD depending on the severity of the inflammation. We reviewed the English literature in the MEDLINE database by searching with "gastrointestinal", "GVHD", and "endoscopy" as key words (Table 1). Although there are some reports of normal mucosal findings, a large number of studies reported findings of erythema, erosions, and edema. Because the symptoms were generally mild in the upper GI tract, the lower GI tract often had relatively severe inflammatory symptoms, with occasional actively bleeding ulcers. In addition, although tortoise shell-like mu-

Table 1 Literature review of the endoscopic findings in gastrointestinal graft-versus-host disease

No.	Ref.	Patients	Involved GI tract	Endoscopic findings
1	Sultan *et al*[23]	Acute GVHD patients less than 18 yr old (n = 40)	Stomach, duodenum, sigmoid and rectum	Normal, erosions, ulcer
2	Kreisel *et al*[24]	GI-GVHD patients (n = 175)	Stomach, duodenum, colon and rectum	Normal, edema, erosions, aptha, ulcer, nonspecific
3	Aslanian *et al*[25]	Acute GVHD patients (n = 18)	Stomach, duodenum, total colon and rectum	Normal, erythema, edema, erosions, ulcer
4	Martínez *et al*[26]	GI-GVHD patients (n = 20)	Stomach, duodenum, colon and rectum	Normal, erythema, edema, erosions, ulcer, bleeding
5	Krishna *et al*[27]	A 49-yr-old man	Stomach, duodenum, colon and rectum	Normal, erosions
6	Al Ashgar *et al*[28]	A 23-yr-old male who had undergone allogeneic HSCT	Sigmoid colon and rectum	Erythema, edema
7	Varadarajan *et al*[29]	Acute GVHD patients (n = 11)	Stomach, duodenum, small bowel, colon and rectum	Normal, erythema, edema, erosions, ulcer, bleeding
8	He *et al*[17]	GI-GVHD patients (n = 32)	Colon and rectum	Erythema, edema, erosions, ulcer, bleeding, tortoise shell-like mucosa, pseudomembrane mucosa
9	Xu *et al*[8]	GI-GVHD patients (n = 8)	Stomach, duodenum, colon and rectum	Normal, erythema, edema, erosions, ulcer, bleeding
10	Cheung *et al*[30]	GI-GVHD patients (n = 44)	Stomach, duodenum, colon and rectum	Normal, erythema, edema, erosions, ulcer, nonspecific, bleeding
11	Ross *et al*[31]	GI-GVHD patients (n = 92)	Stomach, duodenum and rectum	Normal, erythema, edema, erosion, ulcer, nonspecific
12	Harada *et al*[32]	GI-GVHD patients (n = 15)	Colon and rectum	Erythema, edema, erosion, nonspecific
13	Khan *et al*[33]	Acute GVHD patients (n = 38)	Stomach, duodenum, colon and rectum	Edema, erosion, ulcer, bleeding, nonspecific, pseudomembrane mucosa
14	Silbermintz *et al*[34]	An 8-yr-old child	Small bowel	Erosion, bleeding
15	Holmberg *et al*[35]	GI-GVHD patients (n = 90)	Stomach, duodenum, colon and rectum	Normal, erythema, edema, ulcer
16	Onozawa *et al*[36]	An 18-yr-old male	Stomach, duodenum, colon and rectum	Normal, nonspecific
17	Yeh *et al*[37]	GI-GVHD patients (n = 4)	Stomach, duodenum, small bowel, colon and rectum	Erythema, edema, erosions, ulcer, bleeding, nonspecific
18	Schulenburg *et al*[38]	GI-GVHD patients (n = 29)	Stomach, duodenum, colon and rectum	Ulcer, erythema, erosion, normal
19	Cruz-Correa *et al*[39]	GI-GVHD patients (n = 44)	Stomach, duodenum, colon and rectum	Normal, erythema, edema, erosions, ulcer, bleeding, nonspecific
20	Wakui *et al*[40]	GI-GVHD patients (n = 44)	Stomach, duodenum, colon and rectum	Erythema, erosion, ulcer
21	Terdiman *et al*[41]	Acute GI-GVHD patients (n = 12)	Stomach, duodenum, colon and rectum	Normal, nonspecific

GI-GVHD: Gastrointestinal graft-versus-host disease; HSCT: Hematopoietic stem cell transplantation.

cosa and pseudomembrane formation are occasionally observed, no particular findings are reportedly associated with the colorectal mucosa. Moreover, even though some papers report mucosal sloughing and ulceration[8], a wide area of sloughing mucosa along with ulcers and inflamed mucosa, as observed in the present case, has never been reported. We believe that a combination of severe GVHD and CMV infection is the pathogenesis responsible for these symptoms[13]. According to He *et al*[17], GI-GVHD accompanied by CMV infection causes deep, discrete ulcers. The ischemic consequences of occluded blood vessels caused by enlarged vascular endothelial cells due to CMV infection are thought to be the mechanism underlying the GI mucosal damage[18]. Similarly, ischemic alteration, in addition to the cytotoxicity caused by GVHD[19], is a likely cause of the severe symptoms in our patient.

The early and accurate diagnosis of CMV infection in the GI tract is the key to preventing severe symptoms, such as perforation and bleeding[20]. In this case, even though immunostaining with an anti-CMV antibody was negative, the quantitative PCR results of the biopsy specimens were positive, with a high value of 1.0×10^4 copies/μg DNA, which enabled the diagnosis of CMV-GID. The diagnostic accuracy of the quantitative PCR method using biopsy samples is reportedly superior to that of immunostaining to determine the involvement of CMV in the GI symptoms[21]. This case showed that the results of the CMV quantitative PCR were closely correlated with the post-treatment improvement of the mucosa, suggesting the usefulness of the technique for evaluating the effects of CMV treatment.

In conclusion, when endoscopic observation is performed on HSCT patients with postoperative GI symptoms, it is necessary to look for signs of mucosal sloughing and ulcers. In addition to a detailed endoscopic observation, biopsy samples should be examined for the characteristic pathological features of GVHD[22] and for signs of CMV infection. The course of this case suggests that the quantitative PCR of biopsy samples is useful for revealing CMV infection in the GI tract.

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CASE REPORT

Emphysematous cholecystitis with massive gas in the abdominal cavity

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Abstract

Emphysematous cholecystitis is a rare variant of acute cholecystitis with a high mortality rate. The combination of emphysematous cholecystitis and pneumoperitoneum is also rare. We herein describe a case of emphysematous cholecystitis with massive gas in the abdominal cavity. A 77-year-old male presented with epigastric pain and lassitude lasting for one week. A computed tomography scan demonstrated massive gas in the abdominal cavity. Gas was also detectable inside the gallbladder. Massive ascites as well as a pleural effusion were also detected. Under the diagnosis of perforation of the digestive tract, we performed emergency surgery. Beyond our expectations, the perforation site was not in the alimentary tract, but rather in the gallbladder. We then diagnosed the patient with emphysematous cholecystitis with perforation, and performed cholecystectomy. A pathological examination of the resected gallbladder revealed necrosis in the mucosa and thinning of the wall. Cultures of the ascites detected *Clostridium perfringens*, a gas-producing microorganism.

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Key words: Emphysematous cholecystitis; Pneumoperitoneum; *Clostridium perfringens*

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INTRODUCTION

Emphysematous cholecystitis is a type of acute cholecystitis characterized by the presence of intramural and/or intraluminal gas that may develop into gangrene or perforation of the gallbladder. The morbidity and mortality rates of emphysematous cholecystitis are considerable[1]. The disease begins with acute cholecystitis followed by ischemia or gangrene of the gallbladder wall and an infection caused by gas-producing bacteria.

Emphysematous cholecystitis is an uncommon variant of acute cholecystitis. Emphysematous cholecystitis occurring in association with a pneumoperitoneum is very rare. Modini *et al*[2] reported the 16th case of emphysematous cholecystitis with a pneumoperitoneum in the English-language literature in 2008. Thereafter, only one case was reported[3]. We herein report the 18th known case. What is of much note is that, among these cases, the finding of macroscopic perforation of the gallbladder was made in only eight patients[4].

This report presents a case of emphysematous cholecystitis causing a pneumoperitoneum with the finding of macroscopic perforation of the gallbladder.

CASE REPORT

A 77-year-old male was transported to our hospital in September 2011 because he was found falling down in his home with epigastric pain and lassitude lasting for one week. There was nothing particular in the patient's

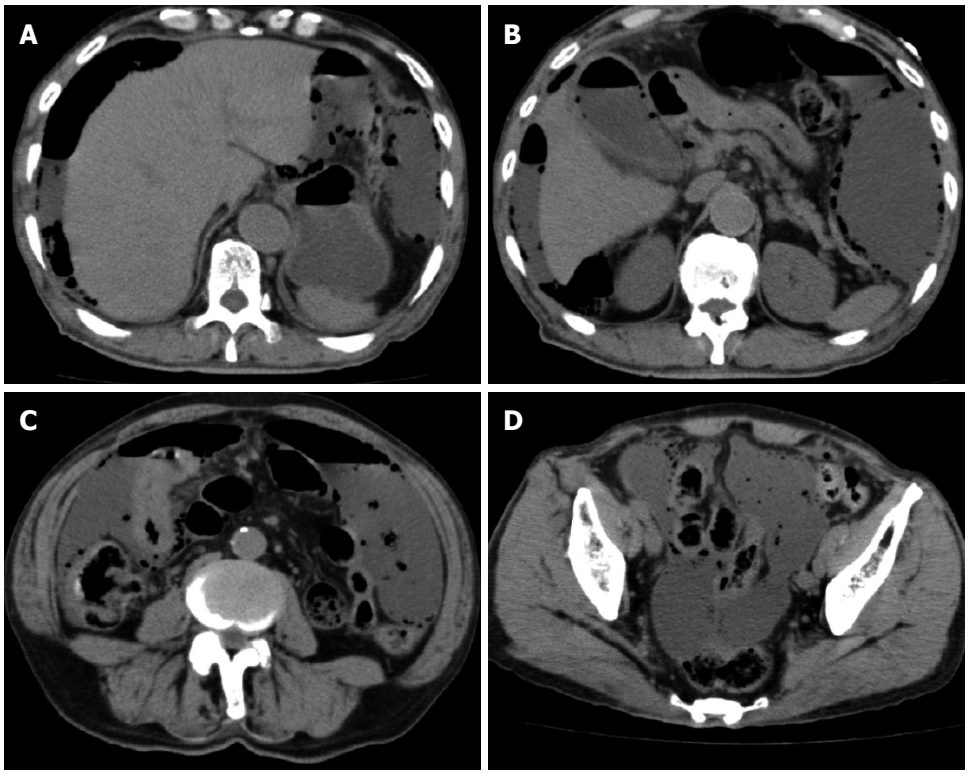

Figure 1 Computed tomography scan (axial axis). A: Demonstrating massive gas in the abdominal cavity; B: Demonstrating gas inside the gallbladder; C, D: Demonstrating massive ascites as well as a pleural effusion.

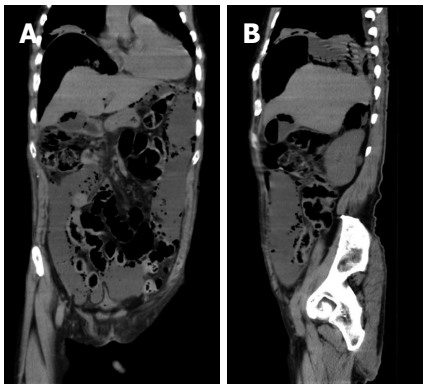

Figure 2 Computed tomography scan clearly demonstrated a massive collection of gas inside the gallbladder. A: The coronal axis; B: Sagittal axis.

history. His vital signs were as follows: pulse: 115 beats/min, blood pressure: 151/86 mmHg, respirations: 36 breaths/min, saturation: 92% on room air, and temperature: 37.7 ℃. The patient's abdomen was distended, and there was local tenderness in the upper abdomen without muscular defense. A laboratory examination showed the following values: leukocyte count: 16.6 × 10^3/μL, hemoglobin: 14.4 g/dL, hematocrit: 42.4%, platelet count: 16.5 ×10^4/μL; serum values: sodium: 130 mEq/L, potassium: 3.8 mEq/L, blood urea nitrogen: 88 mg/dL, creatinine: 3.6 mg/dL, alkaline phosphatase: 251 U/L, lactic dehydrogenase: 371 U/L, aspirate aminotransferase: 32 U/L, alanine aminotransferase: 25 U/L, total bilirubin: 0.4 mg/dL, γ-glutamyl trans ferase: 28 U/L, glucose: 207 mg/dL. Plain abdominal radiography showed the presence of intestinal gas. A computed tomography (CT) scan demonstrated massive gas in the abdominal cavity (Figure 1). Gas was also detectable inside the gallbladder (Figures 1B and 2). Massive ascites as well as a pleural effusion were also detected (Figure 1C and D). Under the diagnosis of perforation of the digestive tract, emergency surgery was performed. In the abdominal cavity, there was massive yellow-brown purulent ascites that did not include any saburra. Contrary to our expectations, the perforation site was not in the alimentary tract, but rather in the gallbladder (Figure 3). We therefore diagnosed the patient to have emphysematous cholecystitis with perforation, and performed cholecystectomy. No gallstones were detected in the gallbladder. Tazobactam/piperacillin was given pre- and post-operation. The patient's postoperative course was uneventful, and he was discharged healthy 28 d after undergoing surgery. Cultures of the ascites detected *Clostridium perfringens*. A pathological examination of the resected gallbladder revealed necrosis in the mucosa and thinning of the wall.

DISCUSSION

Emphysematous cholecystitis is a type of acute cholecystitis characterized by the presence of gas in the gallbladder wall. The disease begins with acute cholecystitis followed by ischemia or gangrene of the gallbladder wall and infection caused by gas-producing bacteria. Whereas the mortality rate of uncomplicated acute cholecystitis is

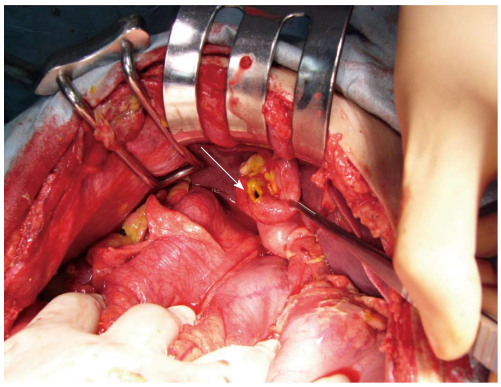

Figure 3 An 8 mm hole in the gallbladder was detected (arrow).

approximately 1.4%, that of acute emphysematous cholecystitis is 15%-20% due to the increased incidence of gallbladder wall gangrene and perforation[5]. Therefore, prompt diagnosis and treatment are essential. The most common symptoms are right upper quadrant pain, low-grade fever, nausea and vomiting. Peritoneal signs may be present, and masses in the right upper quadrant may be palpated in as many as half of patients. CT scanning is the most sensitive test for detecting emphysematous cholecystitis. The presence of gas within the gallbladder wall and lumen is easily confirmed on CT scans.

Emphysematous cholecystitis is more common in males than females (7:3), and 40% of affected patients have diabetes mellitus. In our case, there was no history of diabetes mellitus. After the operation, his blood sugar was decreased down to a normal level, and HbA1c was within normal range.

The presence of a concomitant pneumoperitoneum, which may occur following gallbladder perforation, is rarely found. Most patients with a concomitant pneumoperitoneum are in unstable condition. Therefore, the first choice of treatment in such cases is emergency exploratory laparotomy, followed by cholecystectomy, under a correct intraoperative diagnosis. Another method of treatment, involves initial percutaneous cholecystostomy with a strict intravenous antibiotics regimen, followed by subsequent cholecystectomy during a second stage[4]. In severely ill patients in particular, percutaneous cholecystostomy with broad-spectrum antibiotics may be an alternative choice of treatment[5]. In our case, we did not diagnose the patient with emphysematous cholecystitis preoperatively due to the huge amounts of gas in the abdominal cavity. Compared with previous cases, the amount of gas in our case was very large, thus suggesting not an acute stage, but a sub-acute stage and continuous infection with gas-producing bacteria. If the correct diagnosis could be done preoperatively, laparoscopic surgery may be the alternative treatment.

The bacteria most frequently cultured in this setting include anaerobes, such as *Clostridia welchii* or *Clostridia perfringens*. The bacteria second most frequently cultured include aerobes, such as *Escherichia coli*[5]. In our case, cultures of the ascites detected *Clostridium perfringens*. Zeebregts *et al*[4] reported that, in six of 14 cases, *Clostridium perfringens* was detected on cultures.

In conclusion, we herein reported a case presenting with a very large amount of gas in the abdominal cavity due to gallbladder perforation. We first suspected the possibility of upper gastrointestinal perforation and performed emergency surgery. However, we found a perforation not in the gastrointestinal tract, but rather in the gallbladder. Pneumoperitoneums are nearly always due to perforation of the gastrointestinal tract, however, although unusual, they may also be caused by emphysematous cholecystitis.

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An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Please write a summary of less than 100 words to outline the most innovative and important arguments and core contents in your paper to attract readers.

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For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

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Three-line tables should be numbered 1, 2, 3, *etc*., and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

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Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of *P* values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of *P* values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc*., in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

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English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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