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## Wrap choice during fundoplication

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### Abstract

Gastro-oesophageal reflux disease is an increasing health burden. The mainstay of treatment has conventionally been medical therapy but since the introduction of laparoscopic surgery laparoscopic anti-reflux surgery has been increasingly used for intractable symptoms or in patients unwilling to take long term medication. The Nissen 360 degree wrap has traditionally been considered the gold standard operation but can be associated with significant complications. These complications include "gas bloat" and dysphagia and can occur relatively frequently. Various modifications have been described to the original operation and some of these have been described. In addition alternative wraps have been described which seem to have a reduced incidence of complications associated with their use. This editorial discusses the various types of wrap that can be performed and the minimum requirements of the surgical technique. The evidence from a recent meta-analysis of the randomised data has suggested that an anterior wrap is associated with a lower rate of complications and gives just as good control of reflux symptoms. The advantages and disadvantages of an anterior wrap are discussed. The lack of long term follow up data concerns some practitioners and at the moment the choice of wrap carried out still rests with the individual surgeon.

**Key words:** Fundoplication; Wrap; Laparoscopic; Reflux disease; Choice

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**Core tip:** The type of wrap chosen during a laparoscopic fundoplication will be decided by the surgeon but the evidence suggests that an anterior wrap is associated with less complications than a full posterior wrap and gives just as good control of reflux.

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## INTRODUCTION

Gastro-oesophageal reflux disease (GORD) is a common disorder and the prevalence is increasing. GORD represents a considerable healthcare burden and has significant effects on patients quality of life.

Since the first laparoscopic Nissen fundoplication (LNF) was described in 1991<sup>[1]</sup> there have been many studies of antireflux surgery reported and laparoscopic fundoplication is now an established treatment of GORD<sup>[2]</sup>. Anti-reflux surgery has evolved over the years with increasing practitioner experience and advances in laparoscopic techniques. These advances have led to a safer and more satisfactory outcome for many patients, with a reduction in requirements for open surgery, shorter length of stay and more cost effective healthcare.

## SURGICAL OPTIONS

LNF is considered the gold standard surgical procedure and is the most widely used fundoplication variant for the treatment of gastro-oesophageal reflux disease but it is complicated by a number of unwanted functional disorders such as dysphagia and gas bloat syndrome. These symptoms have been reported to occur at very high rates in the initial post-operative period, although troublesome dysphagia persists in up to 10% of patients at a year<sup>[3-5]</sup> and only between 1% and 10% of patients require reoperation with up to 25% requiring balloon dilation at endoscopy<sup>[4,6]</sup>.

In an attempt to reduce the incidence of these complications post fundoplication a number of modifications and variants have been suggested. Some authors have reported routine division of the short gastric vessels but randomized controlled data has suggested no difference in dysphagia either in the short or long term but has suggested a slight increase in post-operative epigastric bloating<sup>[7,8]</sup>. The consensus of opinion is therefore that the short gastric vessels only need to be divided if it is required to achieve a tension free fundoplication. Leaving the crura untouched and minimal dissection techniques have also been proposed as a means of reducing post-operative dysphagia but these have never been tested in a randomized manner<sup>[9]</sup>. Although the evidence is not strong, most would however advocate hiatal closure if the defect is moderate or large<sup>[10]</sup>. A single randomized study has reported on the use of a 56 French bougie placed in the oesophagus during construction of the fundoplication. This study did report a reduced incidence of dysphagia at a short follow up period, it also reported a small (1.2%) incidence of oesophageal injury secondary to the bougie and on this basis a bougie is not widely used<sup>[11]</sup>.

A number of alternative wraps have been described to try to address the functional problems associated with a full 360-degree Nissen fundoplication. The wrap types can be split into posterior wraps where the stomach is wrapped behind the oesophagus. These include both the Nissen fundoplication and the 270-degree posterior Toupet wrap. Alternatively, anterior wraps where the stomach is passed anterior to the oesophagus such as the 180-200 degree Watson or Dor wraps. Anything less than a 180° wrap has been dismissed as inadequate<sup>[12]</sup>.

Partial wraps were initially reported as advantageous in patients with reduced oesophageal motility and therefore potentially at higher risk of post-operative dysphagia but recent evidence has suggested that this is not necessarily correct<sup>[13,14]</sup>.

The choice of wrap has traditionally been based on anatomic considerations and surgeon preference. The lack of standardization can make comparison of techniques difficult but most surgeons would accept that the following should occur: (1) Crural repair with non-absorbable sutures; (2) Vagal preservation; (3) A mobilization of at least 3cm of intra-abdominal oesophagus; (4) A tension free wrap with or without division of the short gastric vessels; and (5) A 1.5 to 2 cm wrap incorporating the anterior wall of the oesophagus with at least one suture.

## COMPARISON BETWEEN FULL AND PARTIAL WRAPS

There have been a number of randomized controlled trials that have compared the

outcomes of both full and partial posterior<sup>[15,16]</sup> and posterior with anterior fundoplication<sup>[17-19]</sup>. The evidence suggests that there is little difference in post-operative dysphagia when comparing full and partial posterior fundoplication<sup>[20]</sup> and while the evidence from the randomized trials comparing posterior with anterior fundoplication was mixed. A recent meta-analysis has reported that both anterior and posterior fundoplication are equally effective at controlling reflux symptoms the 180° anterior fundoplication is associated with a lower incidence of post-operative dysphagia<sup>[21]</sup>. As a consequence an anterior fundoplication is associated with fewer re-operations (carried out for dysphagia).

In addition to these data, there are technical factors to consider when comparing anterior and posterior fundoplication. There is little doubt that the 180° anterior fundoplication is simpler to perform but the requirement to anchor the fundoplication to the right crus means that re-operation for recurrent symptoms requires more dissection to release the attachments.

The evidence therefore would suggest that the fundoplication associated with the lowest rates of unwanted post-operative symptoms is an anterior 180° fundoplication. This fundoplication will provide an acceptable level of symptom control and patient satisfaction but in the event of the patient requiring further surgery, this type of fundoplication might increase the complexity of that redo surgery. This however, needs to be balanced against the reduce requirement for intervention for post-operative dysphagia.

## CONCLUSION

There is a paucity of long-term follow up data in this field and this has led to practitioners who favour the full posterior fundoplication to question some of the data presented. Ultimately, the decision regarding the type of fundoplication performed will rest with the surgeon and be based on their experience, their preference and the individual patient.

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## Gastric electrical stimulation: An emerging therapy for children with intractable gastroparesis

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### Abstract

Management of gastroparesis remains challenging, particularly in pediatric patients. Supportive care and pharmacological therapies for symptoms remain the mainstay treatment. Although they are effective for mild and some moderately severe cases, often time they do not work for severe gastroparesis. There are a few prokinetics available, yet the use of these drugs is limited by a lack of persistent efficacy and/or safety concerns. Currently, the only modality for adult patients with severe intractable gastroparesis is surgery, *e.g.*, pyloroplasty and partial gastrectomy, however, this option is generally considered too radical for a growing child. Novel therapeutic approaches, particularly those which are less invasive, are needed. This article explores gastric electrical stimulation (GES), a new therapy for gastroparesis. Unlike others, it neither needs medications nor gastrectomy; rather, it treats through the use of microelectrodes to deliver high-frequency low energy electric stimulation to the pacemaker area of the stomach. Thus, it is tolerated and safe in children. Like in adult patients, GES appears to work in releasing symptoms, improving nutrition, and enhancing the quality of life; it also helps wean off medications and eliminate many needs for hospitalization. Considering the transient nature of gastroparesis in children in many occasions, GES is considered a “bridging” therapy after failed medical interventions and before surgery.

**Key words:** Gastroparesis; Gastric electrical stimulation; Nausea; Vomiting; Prokinetics

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**Core tip:** Gastric Electrical Stimulation is an effective, safe and feasible therapy for pediatric patients with symptoms of intractable nausea and vomiting due to medically refractive gastroparesis. It works through the use of microelectrodes to deliver high-frequency low energy electric stimulation to the pacemaker area of the stomach. Even though the mechanism is not completely understood, it provides a “bridge” before radical surgical options and can potentially lead to an improved quality of life by helping in weaning off medications and reducing hospitalizations.

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## INTRODUCTION

Gastroparesis (GP) is defined as a syndrome of objectively delayed gastric emptying in the absence of mechanical obstruction. Symptoms include early satiety, postprandial fullness, bloating, nausea, vomiting, and abdominal pain. Since similar symptomatology may also be seen with other etiologies, including functional dyspepsia, incidence and prevalence is varied. In adults, the age-adjusted prevalence of definite gastroparesis varies from 2.4 to 9.6 per 100000 persons for men and 9.8 per 100000 persons for women<sup>[1]</sup>. In children, the overall prevalence remains unknown, and the data available are limited.

Even though multiple conditions including diabetic, postsurgical and autoimmune causes have been associated with GP, up to 70 % of pediatric cases are idiopathic, 18 % drug-induced and only 12 % are post-surgical<sup>[2]</sup>. Infections have been implicated as a cause of GP in children and can self-recover overtime sometimes as long as 24 mo<sup>[3]</sup>.

In adults, it is standard practice to diagnose gastroparesis utilizing a gastric emptying study as a “gold standard” diagnostic tool but there is no consensus for a standard in pediatrics. A delayed gastric emptying is defined as when a solid meal has a retention of greater than 90% after 1 h, greater than 60% at 2 h, and greater than 10% at 4 h<sup>[4]</sup>. Although the percentage of the patient population having gastroparesis is small, management of it is challenging, particularly in pediatric patients. It often takes a significant amount of time and effort of the treating physician and at times can be frustrating. Patients often require frequent hospitalizations for nutritional support and/or use of multiple medications for symptom control. According to a recent report of 97 pediatric patients followed up to 9.5 years (mean = 3.5 years), all patients had used promotility agents (100%), and most patients were on antiemetic (72%), antireflux (79%), and/or pain meds (52%). Mean hospitalization rates were 3.6 per year. Because of the persistence of symptoms, their quality of life was compromised with frequent school missing or restriction of normal activities. A restricted solid diet was required by 46% and 6% were maintained on a liquid diet only as they were unable to tolerate any kind of solid food. Tube feeding was required by 39% and 4% required parenteral nutrition. Two thirds (66%) of patients had to undergo surgical procedures, however, this latter option is generally considered too radical for a growing child<sup>[5]</sup>. A “bridging” therapy after failed medical interventions and before surgery is needed.

## SUCCESS AND FAILURE OF CURRENT THERAPIES

Supportive care and pharmacological therapies for symptoms remain the mainstay treatment (See [Figure 1](#)). Although they are effective for mild and some moderately severe cases, often times they do not work for severe disease. According to the severity of symptoms, GP can be stratified/graded into three groups: mild, moderate or compensated, and severe or gastric failure<sup>[6]</sup>. Grade 1 (mild) gastroparesis is characterized by intermittent, easily controlled symptoms with the maintenance of weight and nutritional status on dietary modification. Grade 2 (compensated) gastroparesis is characterized by partially controlled symptoms on pharmacological agents and rare hospitalizations. Grade 3 (gastric failure) gastroparesis patients are neither responsive to dietary modification nor to medication, cannot maintain nutrition or hydration *via* the oral route, and require frequent emergency department or inpatient care.

### Dietary modification

It includes eating small, frequent meals of low fat and low fiber diet or liquid diet alleviates the constant feeling of fullness. Diets low in Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols can be effective in some people although the mechanism is not clearly understood.

### Prokinetic agents



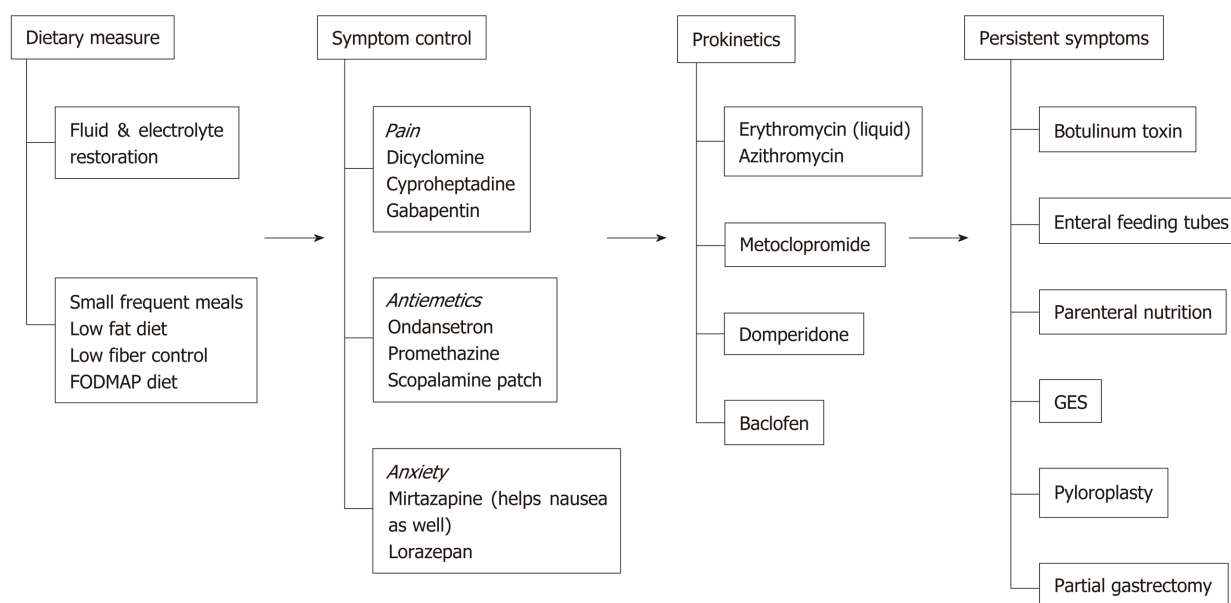


Figure 1 Management of gastroparesis. GES: Gastric electrical stimulation.

Prokinetic agents are medications that enhance gastrointestinal motility and transit of content in the gastrointestinal tract, mainly by amplifying and coordinating the gastrointestinal muscles. A recent literature review talks about the common prokinetic agents used for the management of gastroparesis in pediatrics (Table 1)<sup>[7]</sup>. Metoclopramide is an antagonist of dopamine 2 (D2)-receptor and promotes gastric emptying, as well as binds to the 5-hydroxytryptamine receptor 4 to stimulate cholinergic neural pathways in the stomach. Domperidone works as a D2 receptor antagonist, which enhances antral duodenal contraction and leads to improvement in peristalsis. Through its effect on the chemoreceptor trigger zone, it also exhibits antiemetic properties. These are considered safe and effective drugs in the treatment of gastroparesis, however, there are reports about possible side-effects. Both drugs cause hyperprolactinemia and may cause galactorrhea. Metoclopramide can have extrapyramidal dyskinetic reactions and domperidone can lead to cardiac arrhythmias<sup>[8,9]</sup>. Macrolide antibiotics at reduced antimicrobial dosages, such as erythromycin oral suspension and azithromycin act as motilin agonists and have a significant prokinetic effect. Its safety and efficacy in improving feeding intolerance have been demonstrated in multiple studies with premature infants and children<sup>[10]</sup>. Baclofen, an antispasmodic and muscle relaxant, is another agent that is used in patients with gastroparesis. It probably works by having an inhibitory role on the lower esophageal sphincter relaxation through its stimulation of gammaaminobutyric acid (GABA) B receptors. It also reduces gastric emptying time<sup>[7]</sup>.

### Symptom treatment of nausea, vomiting, pain, and anxiety

Other than prokinetics, the symptomatic treatment of these symptoms remains empirical. These drugs are commonly used off-label from the indications for non-specific nausea and vomiting, palliative care and chemotherapy-induced side effects. (1) Pain is often controlled by medications such as dicyclomine which is an anticholinergic and blocks the action of acetylcholine at parasympathetic sites in smooth muscle. Cyproheptadine is widely used in the pediatric population due to its efficacy in dyspepsia and appetite stimulation<sup>[11,12]</sup>. It is a potent antihistamine and serotonin antagonist with anticholinergic effects. Gabapentin, a GABA analog is reserved for patients with chronic abdominal pain; (2) The most commonly prescribed antiemetic drugs are phenothiazine derivatives and antihistamine agents including promethazine. There is concern about sedation and cardiac toxicity (prolongation of QT)<sup>[13]</sup>. Ondansetron, the 5-HT<sub>3</sub>-receptor antagonist is a reasonable second-line drug for effective nausea control<sup>[14]</sup>, even though it has not been found to be superior to metoclopramide and promethazine in reducing nausea. Transdermal scopolamine is effective for nausea associated with motion sickness and is often used for nausea and vomiting of gastroparesis, although there is no peer-reviewed published report to support this practice; and (3) Symptoms of anxiety may be addressed by the 5-HT<sub>2</sub> receptor antagonist, mirtazapine. It is an atypical antidepressant that works as antiemetic and an appetite stimulant which has been reported efficacious in a single

**Table 1 Synopsis of commonly used prokinetic agents for gastroparesis<sup>[7]</sup>**

Prokinetic agents	Mechanism	Comments/Limitations
Erythromycin	Motilin receptor agonist	QT interval prolongation
Azithromycin		Tachyphylaxis
		Antibiotic and bacteria resistance
Metoclopramide	D <sub>2</sub> antagonist (central/peripheral)	Extrapyramidal symptoms (e.g., tardive dyskinesia, dystonia). Can not use > 12 wk
Domperidone	5-HT <sub>3</sub> antagonist	QT interval prolongation
	5-HT <sub>4</sub> agonist	FDA approved for adults
	Peripheral D <sub>2</sub> antagonist	QT interval prolongation
Baclofen	GABA <sub>B</sub> receptor agonist that inhibits transient lower esophageal sphincter relaxation. Increases gastric emptying	Not approved in United States. Only available through IND
		Muscle weakness, dizziness Very limited data, as trial was limited to a gastroesophageal reflux patients

FDA: Food and drug administration; D<sub>2</sub>: Dopamine receptor 2; 5-HT<sub>3</sub> and 5-HT<sub>4</sub>: 5-hydroxytryptamine receptor 3 and 4; GABA<sub>B</sub>: Gamma-amino-butyric acid B receptor; IND: Investigational new drug.

report in gastroparesis<sup>[15]</sup>. But, like most of the gastroparesis medications, it also prolongs QT<sup>[16]</sup>. Lorazepam, a benzodiazepine has been used for controlling nausea based on anecdotal chemotherapy studies<sup>[17]</sup>

### **Alternative therapies for refractory gastroparesis**

In addition to the aforementioned therapies, there are a few other therapies in use (Table 2).

#### **Endoscopic pyloric botulinum injection**

Botulinum toxin A is a purified neurotoxin that inhibits the release of acetylcholine into the neuromuscular synaptic cleft, which causes a localized reduction in muscle contractility<sup>[18]</sup>. Pediatric data on its use in gastroparesis is limited, however, a retrospective study looked at the endoscopic, submucosal injection of the toxin into the pylorus to reduce the pylorospasm<sup>[19]</sup>. Two double-blind, placebo-controlled studies, showed some improvement in gastric emptying with endoscopic pyloric botulinum injection, but no improvement in symptoms compared with the placebo<sup>[20,21]</sup>.

#### **Enteral feeding**

Children with severe, complicated gastroparesis who are unresponsive to conservative intervention and have had a weight loss of more than 10% due to refractory symptoms of gastroparesis can be offered enteral feeding devices. These are reversible and often temporary. There should be a trial of nasogastric postpyloric feedings prior to jejunostomy feeding tube placement. In 2 large pediatric series, surgical placement of either a gastrostomy or jejunostomy tube was required to aid with management in a small percentage (4%, 19/469) children<sup>[2,3]</sup>. A large study in adults with gastroparesis reported improvement following jejunostomy tube placement with almost 81% patients reporting improvement in overall health status, 56% reporting improved nutrition, 52% with fewer hospitalizations and 39% reporting improved nausea and vomiting<sup>[22]</sup>.

#### **Parenteral nutrition**

Intravenous nutrition is rarely required when hydration and nutritional state cannot be maintained. Enteral feeding should always be preferred over parenteral nutrition for a wide range of practical reasons, such as costs, excessive healthcare utilization and the potential for complications. Parenteral nutrition is also a less “natural” way of delivering nutrition.

#### **Surgical options**

Pyloroplasty & gastrectomy have also been used in the management of gastroparesis. These gastric emptying procedures have been attempted but with limited success in children<sup>[23]</sup>. Moreover, these approaches are permanent and involve the risk of surgical complications, which are not always amenable to the parent or the child. Novel therapeutic approaches, particularly less invasive and reversible “bridging” therapies, are needed after failed medical interventions before surgery.

**Table 2** Alternative therapies for refractory gastroparesis<sup>[2,3,19,21,22]</sup>

Therapy	Mechanism/Indication	Comments
Botulinum Toxin	Endoscopic intra-pyloric injection of botulinum toxin to relax the pylorus	Requires frequent injections No improvement in long term symptoms
Enteral tube feeds	Unintentional loss of 10 % or more of the body weight during a period of 3-6 mo, Refractory symptoms	Mechanical complications: Obstruction, displacement, or dislodgement of the tube. Gastrointestinal complications: formula intolerance, diarrhea, constipation, Hinders normal lifestyle and quality of life
Gastrostomy tube	May be used for venting of secretions to decrease vomiting and fullness	Poor choice for feeding due to delayed gastric emptying
PEG-J tube	Allows the patient to vent gastric secretions to decrease/prevent persistent emesis. Provides jejunal feedings	Migration of the J-tube extension into stomach Pyloric obstruction from J-tube
Jejunostomy tube	Stable access for reliable jejunal nutrient Delivery Avoids gastric penetration	Cannot vent stomach
Dual G and J tube	Two sites-one for venting and one for enteral nutrition	Increased risk of leakage, infection Cosmetic issues
Parenteral Nutrition	Indicated due to intolerance to enteral feeds	Central venous access required. High risk of line infections Time consuming, expensive, and intrusive into daily routines Anesthesia complications
Surgical Options		
Pyloroplasty	Surgical procedure used to widen the pylorus	Radical approach Limited success Surgical and anesthesia complications
Gastrectomy	After failed medical therapy with severe symptoms	Palliative approach Nausea continues to be a problem High risk of surgical and anesthesia complications. Not reversible

PEG: Percutaneous endoscopic gastrostomy; PEG-J: Percutaneous endoscopic gastrostomy with jejunal extension tube.

### ***Gastric electrical stimulation is an emerging “bridging” therapy for pediatric gastroparesis***

In 2000, the United States Food and Drug Administration (FDA) approved the use of Enterra (Medtronic Inc., Minneapolis, MN) gastric electrical stimulator (GES) under the “humanitarian device exemption” for the treatment of diabetic and idiopathic gastroparesis<sup>[24]</sup>. Since then, many prospective cohort studies and randomized controlled crossover studies have been published for adult patients<sup>[25]</sup>. These studies show that GES is effective and safe and can improve the severity of symptoms in adults. However, in pediatrics, we have very limited data with only six published studies so far (Table 3). The largest study has been done in 97 pediatric patients over a period of 10 years. GES was found to be safe and effective in pediatric gastroparesis with continued symptomatic improvement at 1 year and beyond<sup>[9]</sup>.

### ***Understanding of the gastric electrical physiology may lead to the development of novel therapies for treating gastroparesis***

Normal gastrointestinal motor function is a complex series of events requiring coordination of the sympathetic and parasympathetic nervous systems, neurons and pacemaker cells [called interstitial cells of Cajal (ICCs)] within the stomach and the gut. ICCs are the “pacemaker cells” for the smooth muscle apparatus of the gastrointestinal tract. The frequency and direction of the phasic motor activity are regulated by the gastric slow wave, a rhythmic electrical oscillation, which is generated by the ICC located in the upper part of the fundus along the greater curvature. Slow waves are generated in the “pacemaker area” (like the SA node in the heart) and migrate distally to the pylorus at the rate of 3 cycles per minute (cpm) or approximately every 20 seconds. Abnormalities of this process can lead to impairments in gastric emptying. Gastroparesis occurs when ICCs are lost, their activity reduced or conduction blocked. The exact cause of gastroparesis is unknown,

**Table 3 Comparison of pediatric studies on gastric electrical stimulation**

Ref.	Method	Sample Size	Duration	Findings
Islam <i>et al</i> <sup>[28]</sup>	Prospective study on children with chronic nausea and vomiting	9	8-42 mo	7 of the 9 patients reporting sustained improvement in symptoms and improved quality of life
Islam <i>et al</i> <sup>[35]</sup>	Retrospective review in children less than 18 years with diagnosis of gastroparesis	97	10 yr	A significant reduction in all individual symptoms as well as the total symptom score at 1, 6, 12, and 12 mo. Recurrence of symptoms leading to device removal occurred in 7 cases. Forty-one patients had continued improvement in symptoms for over 12 mo, with a mean follow up of 3.5 years
Lu <i>et al</i> <sup>[29]</sup>	Retrospective review on patients with functional dyspepsia	24	6-8 mo	Significant improvements were seen in multiple areas of the PedsQL, including stomach pain/upset, food/drink limits, heartburn/reflux, gas/bloating, patient worry, medication tolerance, and constipation
Teich <i>et al</i> <sup>[35]</sup>	Prospective study on children with chronic nausea and vomiting refractory to medical therapy and met ROME III criteria for functional dyspepsia	16	0.5-23 mo	Significant improvement in severity and frequency of vomiting, frequency and severity of nausea. Also showed decrease in dependence on enteral/parenteral nutrition
Elfvin <i>et al</i> <sup>[36]</sup>	Retrospective review on children with nausea and vomiting	3	12-40 d	Favorable response to temporary percutaneous gastric electrical stimulation with greater than 50% vomiting reduction
Hyman <i>et al</i> <sup>[26]</sup>	Case report on a 7 years old boy with intractable visceral pain and gastroparesis and failure to thrive	1	37 mo	Reduction in pain, retching and vomiting. Successful initiation of enteral feeds and meeting caloric requirements

but the stomach is not paralyzed.

## HOW DOES GES WORK?

GES, although its mechanism is not completely understood, is thought to ameliorate symptoms of nausea and vomiting by improving gastric accommodation. This is done via stimulation of the enteric nervous system in addition to central effects mediated through the vagus nerve. Like in the heart, an external pacemaker, a medical device that generates electrical impulses delivered by electrodes to stimulate and restore the function of the stomach, can be used. Currently, there are two approaches to stimulate the function of the stomach: (1) Gastric pacemaker, which delivers low-frequency high energy electrical stimulus timed to synchronize gastric rhyme (*e.g.*, 3 cycles/min in the gastric antrum and 12 cycles/min in the duodenum) and stimulate muscle movement. This is an ideal method yet is in a developing stage, as there is no implantable power source at present; and (2) GES in which the current uses low energy and high frequency. It does not alter motility patterns<sup>[26]</sup>. This is the method in current use.

### Procedure

Similar to a cardiac pacemaker, the gastric electrical stimulator is an implantable device. It contains an electronic circuit and a battery and is implanted subcutaneously in the abdominal wall. The electrical pulses delivered by the stimulator are provided by the electronic circuit, and the battery provides the energy needed for 5 to 10 years of operation. Both battery and electronic circuits are encapsulated in a titanium

housing, referred to as the pulse generator unit<sup>[26]</sup>. The procedure has been detailed in previous studies<sup>[27,28]</sup>. In a nutshell, placement of temporary GES (tGES) electrodes is carried out under direct visualization using endoscopy. One wire lead is secured into the stomach mucosal wall. This wire exits from one nostril and is connected to the pulse generator which is secured on the outside of the body. A minimum of 3-days with the tGES is required to determine if the individual patient will benefit from a permanent GES. In the permanent GES, a tiny pulse generator and two-wire leads with small electrode ends are surgically implanted in the stomach. Typical initial GES settings are started at 5-7 volts, 14 Hz frequency, 1 second “on” and 4 seconds “off”, and pulse width 330 microseconds with impedance in the range of 400-800  $\Omega$ .

## GES THERAPY HAS ADVANTAGES FOR CHILDREN WITH GASTROPARESIS

It provides a “bridge” treatment option before the radical life-altering surgical options. Unlike other therapies, GES neither needs medications nor gastrectomy; rather, it treats through the use of microelectrodes to deliver high frequency low energy electric stimulation to the pacemaker area of the stomach. Based on the 6 published studies summarized in Table 3, GES is feasible, tolerable and safe in children. Like in adult patients, GES improves symptoms in children, improves nutrition, and enhances the quality of life; it also helps wean off medications and eliminate many needs for hospitalization.

### ***GES helps improve gastrointestinal symptoms in children with gastroparesis***

(1) It reduces overall symptoms in as many as 69% of children, male or female, age 2-18 years with chronic gastroparesis. The most dramatic improvements were emesis score (baseline *vs* GES 1 mo *vs* GES 12 mo = 2.8 *vs* 0.4 *vs* 0.3) and bloating score (2.2 *vs* 0.4 *vs* 0.9), followed by pain (3.6 *vs* 0.9 *vs* 1.6), nausea (3.8 *vs* 1.1 *vs* 1.6), and satiety (2.6 *vs* 0.9 *vs* 1.2)<sup>[5]</sup>; (2) There is a substantial drop in medication use after GES therapy. Most notably in the use of antiemetics (68% *vs* 44%), and prokinetics (41% *vs* 2%). However, there is neither a difference in the use of pain medications (opioids, non-opioids, and neuropathic medications) nor in antireflux medications<sup>[5]</sup>. Another pediatric study showed a decrease in the use of medications after GES<sup>[29]</sup>; (3) Up to 85% of patients report a significant quality of life improvement<sup>[5]</sup>; (4) There is a threefold reduction in tube feed requirements (46% to 13 %) and a two-fold reduction in the need for parenteral nutrition (25% to 13 %) with the application of GES<sup>[30]</sup>. Thus, the use of GES helps transition to normal oral feed; (5) There is a reported reduction in the total number of hospitalizations (5% to 2.9%) for a year for all the associated comorbidities and complications arising from other modalities of treatment<sup>[5]</sup>. This, in turn, means reduced costs for both patient and the healthcare network; and (6) Islam *et al*<sup>[5]</sup> also showed the nutritional effects of GES are related primarily to decrease GI symptoms. These were accompanied by increased weight and body mass index, an improvement in pancreatic function, increased albumin, and a decreased need for enteral tubes or parenteral nutrition. As a result, only 15% (*vs* 46% before GES) patients required using a restricted solid diet and no patient (*vs* 6% before GES) needed to maintain on a liquid diet.

### ***GES is feasible and safe in children with gastroparesis and causes few complications***

**Feasibility and tolerability:** GES has been placed in patients with a mean duration of symptoms of 3.5 years but has been put as early as one month as well. It is found to be most successful in female adolescents but has also been used in children as young as 2 years of age<sup>[5]</sup>. At least 4 institutions in the world have reported using this modality to treat over 100 pediatric patients with gastroparesis (Table 3) of varying etiology. Both temporary and permanent GES have been used. The former is placed for only a few days, whereas the latter is for months and years. The longest duration of GES placed is over a 9-year period and was performed by Islam *et al*<sup>[5]</sup> from the University of Florida. During this period, GES remained to function properly without events except for battery replacement. Thus, GES is feasible and well-tolerable in children.

**Safety and complications:** GES is also safe in children and causes few complications. It has been found to be relatively safe in both short term and long term use in the pediatric population with a complication rate of 16% as reported by Islam *et al*<sup>[5]</sup> and 20% as reported by Lu *et al*<sup>[29]</sup> with abdominal pain being the commonest complication. Most of these complications were easily fixed as they are relatively benign such as battery replacement, replacement of electrodes but about 8% had to



have the stimulator explanted and 2% developed an infection. This data seems to reflect better tolerability than adults. Long term tolerability has been reported as long as 3.5 years in children, although the effectiveness seems to plateau and/or decrease after 12 months<sup>[5]</sup>. The maximum effectiveness is apparent with a decrease in symptoms with the temporary stimulator and at the 1-mo mark and even though it tends to wear off as time progresses, the cumulative symptom score is better when compared to baseline.

### **Some unanswered questions**

**What if GES is removed after symptom improvement?** Few data are available and more studies are needed to address this question. However, in their study, Islam *et al*<sup>[5]</sup> observed two patients whose symptoms remained in remission for 2-3 years following GES removal although disease relapsed eventually in both cases.

**Who responds to GES? Can responders be predictable?** Future studies are needed. Based on current data, it appears that nausea/vomiting-dominant gastroparesis responded better than pain-dominant gastroparesis. Those with narcotic-dependence or with a history of psychosis or eating disorders tended to respond poorer than those without. Female adolescents seem to have responded the best.<sup>[25]</sup>

**Do we really understand the mechanism behind it?** The mechanism behind the improvement seen with GES therapy remains poorly understood. Systematic reviews reveal that gastric electrical stimulation has not been shown to affect gastric emptying in a consistent manner. It does not appear to entrain gastric muscle, either. The observations that GES works better for symptoms of nausea and vomiting and not in pain predominant constellation of symptoms and that it does not accelerate gastric emptying time even after symptomatic improvement<sup>[31]</sup> seem to suggest that GES may improve symptoms centrally through altering central nervous system control of nausea and vomiting via the gut-brain axis. Indeed, human studies before and during gastric stimulation have shown increases in EGG amplitude, vagal activity, and positron emission tomography-imaged activity in the thalamic and caudate nuclei of the brain during chronic GES, yet the demonstration of effect centrally does not imply causation, because the alterations in propagation velocity with GES may reflect enteric and/or autonomic changes induced by electrical stimulation<sup>[32]</sup>. Alternatively, GES may improve symptoms by increasing gastric accommodation, as revealed by Xing JH, *et al*<sup>[33]</sup>. Finally, a cross-over study done on patients with gastroparesis placed with GES showed that the symptoms did not significantly improve in blinded ON vs. Off (Phase I) but did in open-label (Phase II), suggesting that there may also be a component of a placebo effect<sup>[34]</sup>. Large randomized controlled trials are needed to verify this.

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## **TAKE HOME MESSAGE**

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GES can be a suitable option for children and adolescents with medically refractory gastroparesis, even though currently GES is used as an “off label” indication, it can lead to long-term significant improvements in all symptoms that translates to a sustained decreased medication usage, the number of hospitalizations, healthcare costs and improved quality of life.

The system is not risk-free; however, it provides a better alternative to permanent enteral feeding tubes, central lines for parenteral nutrition and overuse of medications. This may be a more cost-effective solution in the long run for patients with medically refractory gastroparesis as it decreases then need for medications, hospitalizations, specialty formulas, etc. Gastric Electrical Stimulation helps the patient return to a more “normal” daily routine and even though some studies consider it to have a placebo effect, it is worthwhile in children and adolescents who are otherwise a very vulnerable group.

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## **CONCLUSION**

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We think if GES is approached in a systematic manner, some of the potential complications can be averted in children who then can be provided with a more long-term symptom-free solution. Although gastric pacing may be the answer to such a complex disease syndrome, it is currently in the stage of infancy in terms of development for practical use. GES provides a bridge therapy option for medically refractory gastroparesis and a pediatric gastroenterologist should be aware of this new relatively unexplored modality of treatment. Finally, GES is a reversible

procedure and carries no mortality risk.

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## Basic Study

# Comprehensive multi-omics analysis identified core molecular processes in esophageal cancer and revealed GNGT2 as a potential prognostic marker

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## Abstract

### BACKGROUND

Esophageal cancer is one of the most poorly diagnosed and fatal cancers in the world. Although a series of studies on esophageal cancer have been reported, the molecular pathogenesis of the disease remains elusive.

### AIM

To investigate comprehensively the molecular process of esophageal cancer.

### METHODS

Differential expression analysis was performed to identify differentially expressed genes (DEGs) in different stages of esophageal cancer from The Cancer Genome Atlas data. Extracting gene interaction modules were generated, and hub genes in the module interaction network were found. Further, through survival analysis, methylation analysis, pivot analysis, and enrichment analysis, some important molecules and related functions/pathways were identified to elucidate potential mechanisms in esophageal cancer.

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## RESULTS

A total of 7457 DEGs and 14 gene interaction modules were identified. These module genes were significantly involved in the positive regulation of protein transport, gastric acid secretion, insulin-like growth factor receptor binding, and other biological processes as well as p53 signaling pathway, epidermal growth factor signaling pathway, and epidermal growth factor receptor signaling pathway. Transcription factors (including hypoxia inducible factor 1A) and non-coding RNAs (including colorectal differentially expressed and hsa-miR-330-3p) that significantly regulate dysfunction modules were identified. Survival analysis showed that G protein subunit gamma transducin 2 (GNGT2) was closely related to survival of esophageal cancer. DEGs with strong methylation regulation ability were identified, including *SST* and *SH3GL2*. Furthermore, the expression of *GNGT2* was evaluated by quantitative real time polymerase chain reaction, and the results showed that *GNGT2* expression was significantly upregulated in esophageal cancer patient samples and cell lines. Moreover, cell counting kit-8 assay revealed that *GNGT2* could promote the proliferation of esophageal cancer cell lines.

## CONCLUSION

This study not only revealed the potential regulatory factors involved in the development of esophageal cancer but also deepens our understanding of its underlying mechanism.

**Key words:** Esophageal cancer; Molecular pathogenesis; Enrichment analysis; Gene interaction module; Regulatory factors; *GNGT2*

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**Core tip:** Based on the esophageal cancer-associated RNA-seq in The Cancer Genome Atlas, we studied differentially expressed genes of esophageal cancer at various stages, constructed a protein-protein interaction network, obtained 14 dysfunctional modules, and screened Hub genes. We performed enrichment analysis to predict non-coding RNA and transcription factors as well as methylation analysis of the genes in the module. A series of regulatory factors was predicted to regulate to a certain degree the potential dysfunction mechanism of esophageal cancer, which provides new insight for future studies of esophageal cancer.

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## INTRODUCTION

Esophageal cancer is one of the world's most common cancers with poor diagnosis and high mortality because of invasiveness and a fast growth rate<sup>[1]</sup>. From a therapeutic point of view, esophageal cancer can be divided into early esophageal cancer, locally advanced resectable esophageal cancer, locally advanced unresectable esophageal cancer, and metastatic esophageal cancer. Because of the anatomical features of esophageal cancer, esophageal cancer is usually detected in the late stage, which vitally affects the treatment options and prognosis of patients<sup>[2]</sup>.

During the development of esophageal cancer, the rs11473 polymorphism of the miR-483-5p binding site plays a vital role in the 3'-untranslated region of the basigin gene<sup>[3]</sup>. Single nucleotide polymorphisms in telomerase reverse transcriptase may be associated with susceptibility to esophageal cancer and contribute to the development of esophageal cancer<sup>[4]</sup>. MiR-20b may play an essential role in the tumorigenesis of esophageal cancer by regulating phosphatase and tensin homolog expression, which may be a potential therapeutic target for the treatment of esophageal cancer<sup>[5]</sup>. Growing evidence has revealed molecular targets for diagnosis and prognosis using



bioinformatic analysis in the field of oncology<sup>[6-16]</sup>. These findings have deepened our understanding of the pathogenesis of esophageal cancer and have guided the direction of our research. However, the molecular pathogenesis of the disease is still elusive.

To explore comprehensively the molecular processes and potential therapeutic targets of esophageal cancer progression, we conducted a systematic module analysis. Overall, our work details the role of multifactorial mediated dysfunction modules in the growth of esophageal cancer and identifies essential genes and related biological processes, finding potential molecular mechanisms and therapeutic targets [G protein subunit gamma transducin 2 (GNAT2)] for esophageal cancer.

## MATERIALS AND METHODS

### *Patient samples and cell lines*

All esophageal cancer analyses in this study involving human participants were in accordance with the ethical standards of the Second Hospital of Jilin University and with the Declaration of Helsinki. A total of 40 esophageal cancer patients and healthy control volunteers, who matched for age and sex, were involved in this study. Informed consent was obtained from all participants. The esophageal cancer cell lines EC109 and KYSE70 were kindly provided by Laboratory in The Second Hospital of Jilin University. The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum.

### *Quantitative real-time PCR and cell proliferation experiment*

Total RNA was extracted from case/control group using TRIzol. The quantitative real time-polymerase chain reaction (PCR) experiment was conducted in a real-time PCR detection system using SYBR Green qPCR Master Mix. Primers were designed and synthesized by Novogene (Beijing, China). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. Cell counting kit-8 assay was used to measure cell proliferation. Transfected cells were cultured for 0-96 h and incubated at 37 °C for 2 h. A spectrophotometer (450 nm) was used to quantitate samples.

### *Data resource*

The Cancer Genome Atlas (TCGA) is a joint project of the National Cancer Institute and the American Human Genome Research Institute. High-throughput genomic analysis technology is a useful tool for people to understand better cancer, and it improves their abilities to prevent, diagnose, and treat disease. We downloaded esophageal cancer RNA-Seq data from the TCGA database and screened non-coding RNA (ncRNA)-mRNA interaction pairs with a score  $\geq 0.5$  from RNA Associated Interaction Database v2.0<sup>[17]</sup>, including 431937 interacting pairs involving 5431 ncRNAs. All human transcription factor target data were downloaded and used in the general database-Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining v2 database for transcriptional studies, including 2492 transcription factors and 9396 interaction pairs.

### *Differential expression analysis*

In order to explore the molecular process of esophageal cancer staging, we selected four stages of esophageal cancer and normal samples for differential expression analysis, including healthy tissue samples *vs* stage 1 disease samples, stage 1 disease samples *vs* stage 2 disease samples, stage 2 disease samples *vs* stage 3 disease samples, and stage 3 disease samples *vs* stage 4 disease samples. We used the limma package for analysis<sup>[18-20]</sup>. Using the Correct background function, we performed background correction and normalization on the data. The normalize Between Arrays function quantile normalization method can filter out the control probe and the low expression probe. The differentially expressed genes of the data set were identified based on the lmFit and eBayes functions ( $P < 0.01$ ) using default parameters.

### *Establishing a protein interaction network to identify esophageal cancer related functional modules*

A protein-protein interaction system was constructed based on Search Tool for the Retrieval of Interacting Genes/Proteins database data (score  $> 500$ ). The gene module of more than 30 nodes was screened throughout the network using the ClusterONE plug-in<sup>[18]</sup> of the Cytoscape software<sup>[19]</sup>. We use the Cytoscape plugin CytoHubba<sup>[20]</sup> to identify hub genes in the module subnet, while CytoHubba contains 12 methods for identifying hub genes. We obtained the top 10 genes and then screened the repeat genes in the 12 sets of genes for survival analysis.

### Enrichment analysis

The study of the functions and signal transduction pathways involved in genes contributes to our understanding of the molecular mechanisms of disease. Gene ontology function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the R language Cluster profiler package<sup>[21]</sup>. The Cluster profiler is a bioconductor software package that provides statistical analysis of functional clustering of gene sets.

### Predictive transcriptional factors and ncRNAs for significant regulatory modules

The transcription and post-transcriptional regulation of genes are often dominated by the regulation of transcription factors (TF) and ncRNA. Therefore, we have scientifically predicted its role in the esophageal cancer dysfunction module. If the regulatory effect between the regulator and the module exceeds 2, and the number of organizational relationships between the regulator and the module is essential (hypergeometric test,  $P$  value  $< 0.01$ ), it can be confirmed that it is a regulator of the critical regulatory module.

## RESULTS

### Identifying the expression of dysregulated molecules in esophageal cancer

Biologists have conducted many experiments and studies on the pathogenesis of esophageal cancer and have thus identified potential pathogenic genes for the deterioration of esophageal cancer. To observe molecular changes in the progression of esophageal cancer, we performed differential expression analysis based on RNA-Seq data from four stages of esophageal cancer in the TCGA database. Based on analysis of phase 1 disease samples of normal tissue samples and esophageal cancer, analysis of phase 1 disease samples and stage 2 disease samples, analysis of stage 2 disease samples and phase 3 disease samples, and analysis of stage 3 disease samples and stage 4 disease samples, we obtained differential expression genes (DEGs) associated with each stage of esophageal cancer. A total of 7457 differentially expressed genes were received (Figure 1B). We believe that the presence of these differentially expressed genes is closely related to the development of various stages of esophageal cancer. Of the 7457 DEGs, there were 13 common genes (Table 1). The genes that were continuously down-regulated are *CPLX2*, *DPEP1*, *EPHA5*, *SCGB1A1*, *ST18*. The genes that were continuously up-regulated are *FGF14*, *KCNH6*, *LOC100506136*, *RGS7*, *SH3GL2*, *THBS4* (Figure 1A).

### Identify functional esophageal cancer staging related modules

Gene module analysis helps us to study the complex collaborative relationships between multiple genes. Based on the protein interaction data of the STRING database, the interaction network of differentially expressed genes was constructed, and 14 functional barrier modules were explored. Using the 12 methods in Cyto-hubba, a total of 758 hub genes were identified in the interaction sub-network of the module genes, including the gene *SH3GL2*, which is continuously up-regulated in Module 8. Further, 23 hub genes shared by the top10 gene set in 12 methods were screened for survival analysis. The results show that *GNGT2* in module 6 is the related gene ( $P = 0.014$ ) (Figure 2A). A decrease in survival rate accompanied the high expression of *GNGT2* gene, and the expression level of *GNGT2* gene was negatively correlated with survival rate. Function and pathway are essential mediators of the physiological response of the disease. We performed GO enrichment analysis on 14 module genes (Figure 2B) and KEGG (Figure 2C). The main biological processes include positive regulation of protein transport, gastric acid secretion, and insulin-like growth factor receptor binding. The main signal transduction pathways involved are the p53 signal transduction, the epidermal growth factor signal transduction, and the epidermal growth factor receptor signal transduction pathways. These pathways play crucial roles in the dysfunctional module for the functions and pathways involved in multiple genes.

### TFs and ncRNAs that drive esophageal cancer progression

From the perspective of systems biology and systems genetics, transcription and post-transcriptional regulation of genes have long been recognized as crucial regulators of disease development, while transcription factors and ncRNAs are universal regulators of expression and function. Although the role of TFs and ncRNA regulation of esophageal cancer progression has been evaluated by many biologists, few studies have focused on their overall global effect on dysfunctional mechanisms and the role they play in development. Therefore, in this study, based on the targeted regulation

Table 1 Differential expression of 13 common genes in four different stages of esophageal cancer

Common genes	DEG_S1	DEG_S2	DEG_S3	DEG_S4
	logFC	logFC	logFC	logFC
CPLX2	-4.85047	2.288678	1.290368	-4.17468
DPEP1	-1.33466	2.340936	1.604402	-4.41133
EPHA5	-6.3307	1.829821	0.991225	-3.72048
FGF14	-2.70845	1.254105	1.339303	-2.01758
INSM1	-2.80099	1.759985	2.787167	-3.27386
KCNH6	-3.87431	1.636848	1.717225	-2.87864
LOC100506136	0.757741	0.789295	-0.58427	0.955725
RGS7	-3.27316	2.095696	1.45118	-3.13742
SCGB1A1	-3.02471	3.325037	-1.81283	-2.46682
SH3GL2	-6.28822	2.761197	1.269058	-2.63088
SLITRK1	-4.12073	2.309863	1.635813	-3.57252
ST18	-2.02067	1.550856	1.289301	-2.51987
THBS4	-2.15432	2.753388	1.06135	-2.2027

DEG\_S: Differential analysis of four different stages *vs* control.

relationship between TF and ncRNA on the module gene, we performed a pivotal analysis of the conventional module to explore the crucial regulator that regulates the progression of esophageal cancer. The results showed that a total of 54 transcription factors involved 54 TF-module target pairs and 853 ncRNAs involved 944 ncRNA-module regulatory pairs. Statistical analysis revealed that TF HIF1A and ncRNA CRNDE regulate the most dysfunctional modules. These crucial transcription factors and ncRNAs may influence the development and progression of esophageal cancer by mediating dysfunctional modules. Thus, we identified these potential factors as regulators of dysfunction in esophageal cancer. Notably, hsa-miR-330-3p up-regulates the differentially expressed gene *SH3GL2* throughout the esophageal cancer process, suggesting that hsa-miR-330-3p plays a crucial role in four stages of esophageal cancer.

**GNGT2 expression is upregulated in the esophageal epithelial cells of esophageal cancer and cell lines**

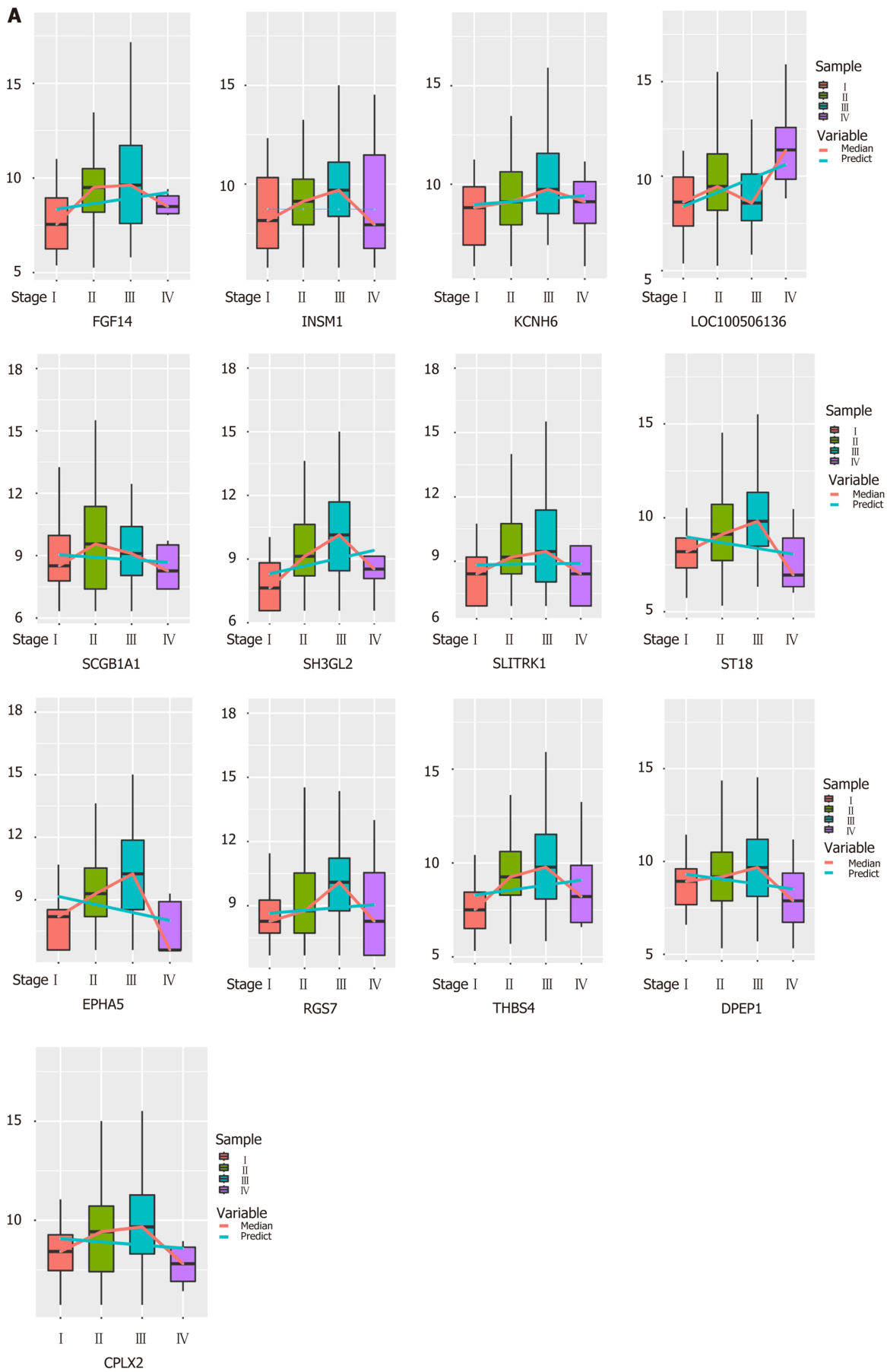
To investigate changes in *GNGT2* expression during esophageal cancer development, samples from esophageal cancer patients (*n* = 20) and esophagus controls (*n* = 20) were subjected to quantitative real time-PCR analysis. As shown in **Figure 3A**, expression of *GNGT2* gene was significantly upregulated in the esophageal epithelial cells of esophageal cancer patients. The experiment in esophageal cancer cell lines (EC109 and KYSE70) showed consistent results (**Figure 3B**) (*P* < 0.05).

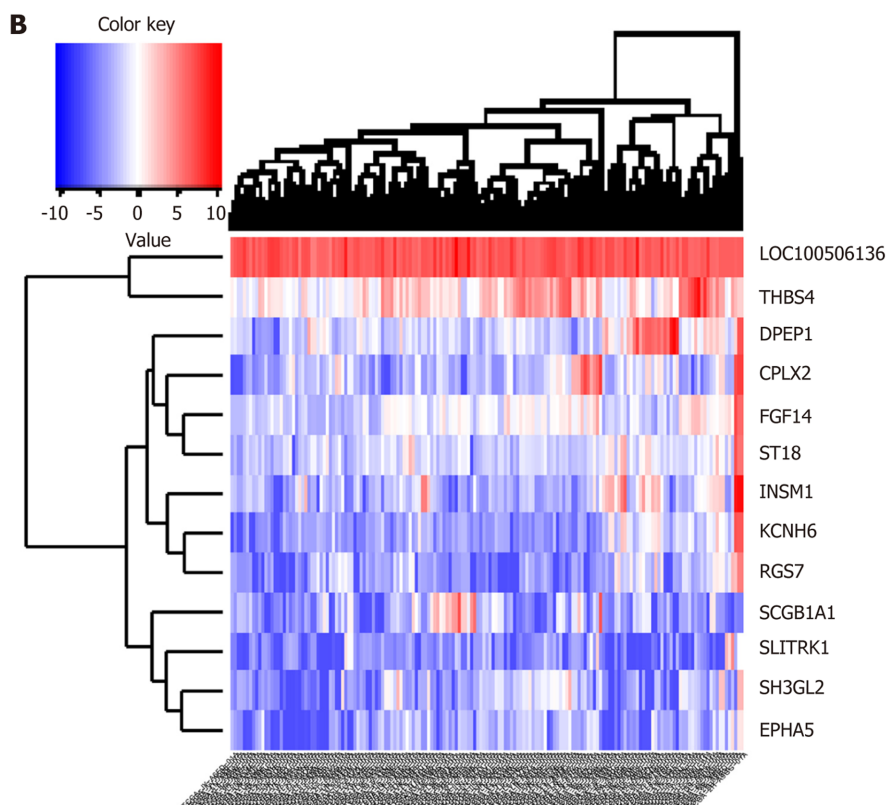
**GNGT2 could promote the proliferation of esophageal cancer cell lines**

To explore further the role of *GNGT2* in the proliferation of EC109 and KYSE70 cells, cells were transfected with *GNGT2* siRNA. As shown in **Figure 3C**, *GNGT2* mRNA expression level was significantly decreased in EC109 and KYSE70 cells. Moreover, the proliferation of *GNGT2* transfected group was significantly lower than that of the control group (**Figure 3D**). Taken together, the above results demonstrated that *GNGT2* could promote the proliferation of esophageal cancer cell lines.

**DISCUSSION**

Esophageal cancer is one of the most deadly cancers, mainly because it is extremely aggressive and has a poor survival rate. Its 5-year survival rate is about 15%-25%<sup>[1]</sup>. The underlying cause of this disappointing low survival rate is that most patients have reached the late stage of disease at the time of detection. For patients with metastatic and unresectable disease, their chances of survival are limited<sup>[22]</sup>. In the present study, we collected RNA-Seq data from TCGA esophageal cancer and selected four stages of esophageal cancer disease samples and normal samples for differential analysis, and obtained four sets of time series differentially expressed genes. After



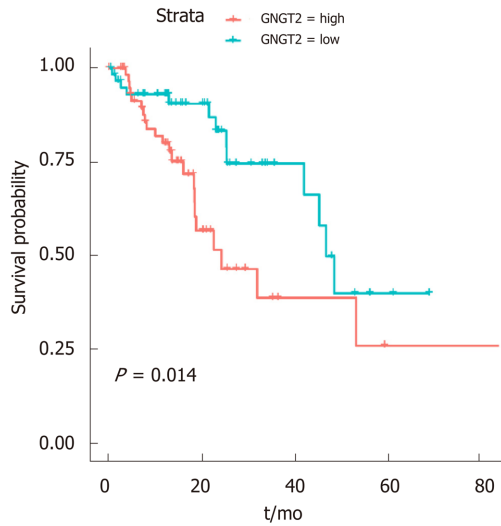
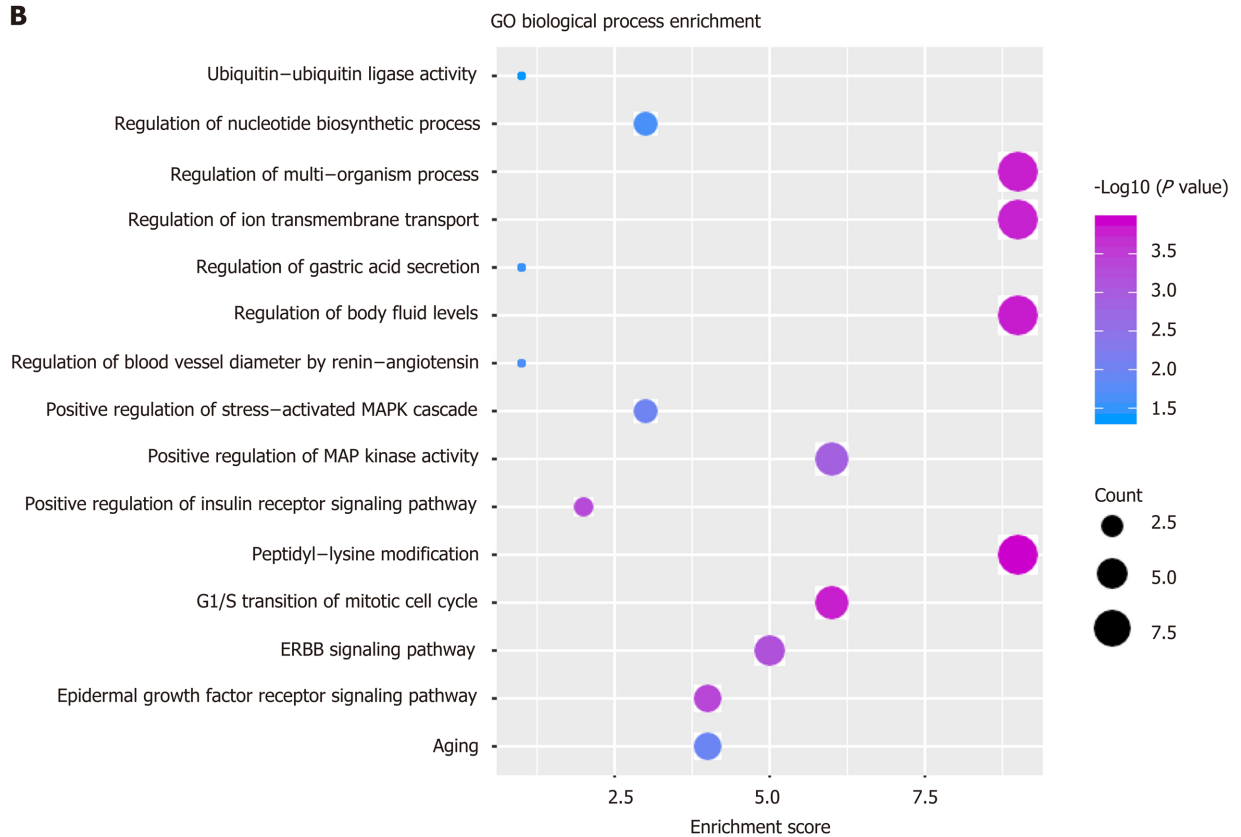


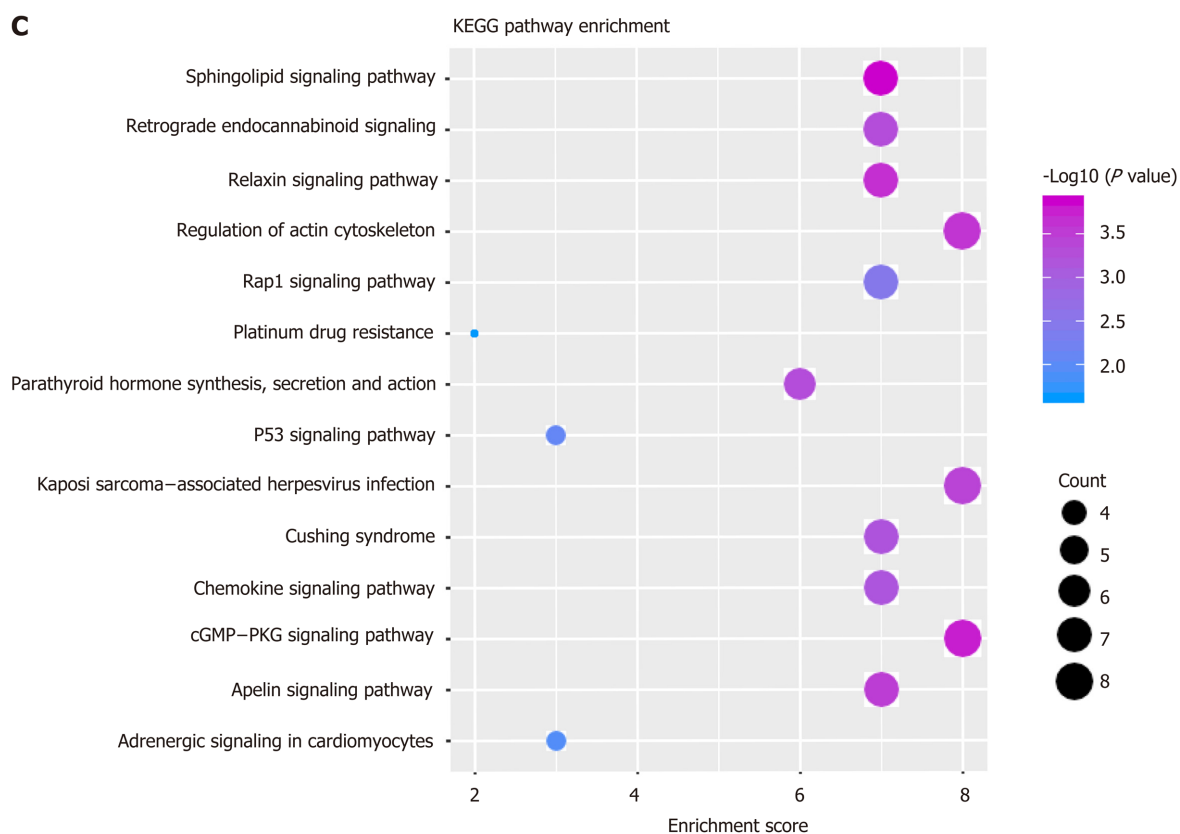
**Figure 1 Synergistic expression of differential genes in four samples of esophageal cancer in patient samples.** A: Continuous regulation of common genes in differentially expressed genes in four stages; B: Expression heat map of common genes in samples of differentially expressed genes in four stages.

screening, we found 13 common genes in four groups of DEGs. Komatsu *et al*<sup>[23]</sup> studied clinical biomarkers of pulmonary neuroendocrine tumors (LNET) and found that CPLX2 was strongly positive in 16.3% of the examination groups. Importantly, positive CPLX2 expression is associated with lymphatic invasion, pathological staging, and adverse disease-specific survival in LNET patients. It was concluded that CPLX2 is a novel clinical biomarker for LNET<sup>[23]</sup>. In the study of breast cancer diagnostic markers, Fu *et al*<sup>[24]</sup> found that changes in gene expression, such as DPEP1, may lead to cancer progression. DPEP1 has been identified as a prognostic gene for colorectal cancer (CRC). We found that DPEP1 is overexpressed in CRC. After knocking out the DPEP1 gene, cells (SW480 and HCT116) exhibited increased apoptosis and attenuated cell proliferation and cell invasion<sup>[25]</sup>. In the study of CRC, Eisenach<sup>[26]</sup> found that the expression of DPEP1 was increased in CRC tissues compared with normal mucosa. Zhang *et al*<sup>[27]</sup> also noted the DPEP1 gene in the study of pancreatic ductal adenocarcinoma and found that its gene expression was negatively correlated with histological grade and that lower expression of DPEP1 in tumors was associated with poor survival. Chen *et al*<sup>[28]</sup> analyzed the gastric cancer-associated Gene Expression Omnibus data and found that thrombospondin 4 (THBS4) was up-regulated in patients with recurrent gastric cancer and was positively correlated with the pathological stage and poor prognosis of gastric cancer. THBS4 stimulates the proliferation of gastric cancer cells. The breast-related gene explored by Huang *et al*<sup>[29]</sup> contains the gene THBS4, which is up-regulated in breast cancer. In the study of hepatocellular carcinoma, Su *et al*<sup>[30]</sup> found that knockdown of THBS4 inhibited migration and invasion of hepatocellular carcinoma cells as well as hemangioma-induced angiogenesis. THBS4 as a target is very promising for the treatment of advanced liver cancer. Both of the above genes were present in the differential genes of the four stages of esophageal cancer in this study and were continuously down-regulated. Moreover, THBS4 was identified as a clinical biomarker gene and a therapeutic target gene in various cancers. Therefore, we can reasonably speculate that this gene plays an important role in the occurrence and development of esophageal cancer, providing a reasonable direction for further study of esophageal cancer.

The results of the methylation test showed that the SST gene was up-regulated extensively, which may be a key gene involved in methylation modification to regulate the progression of esophageal cancer. Jin *et al*<sup>[31]</sup> found that hypermethylation

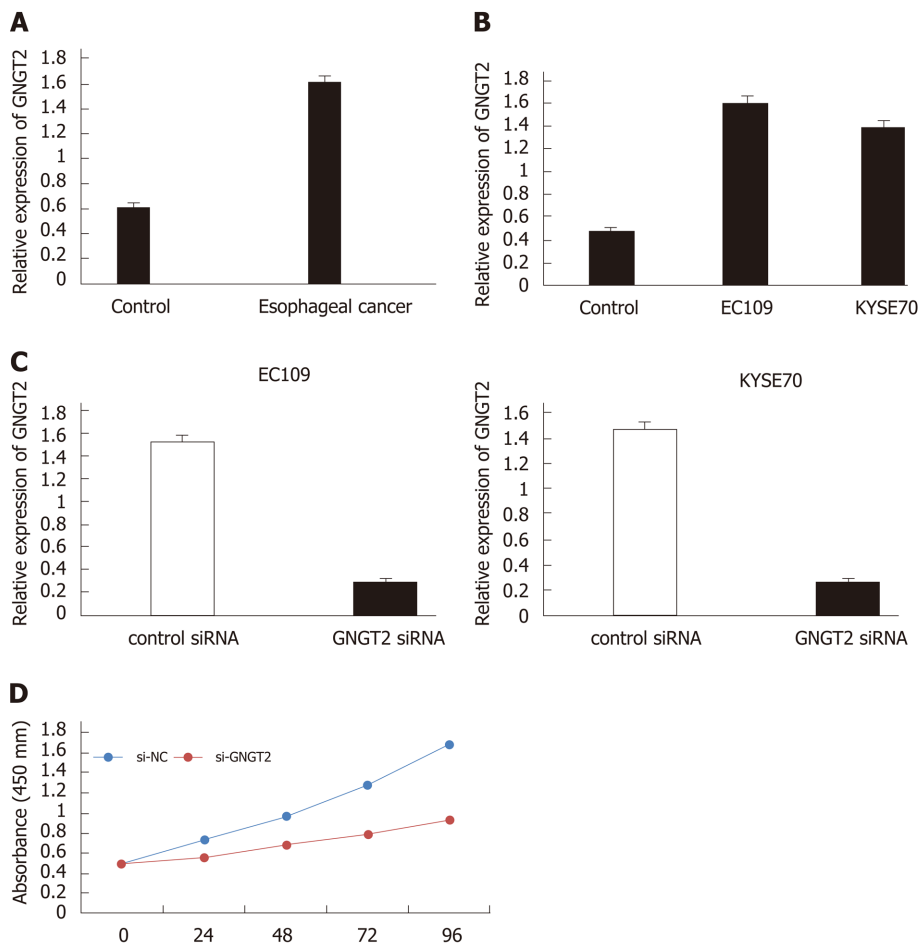


**A** Survival analysis of GNGT2**B**



**Figure 2 Dysfunctional modules.** A: Survival analysis of G protein subunit gamma transducin 2; B and C: Module gene function and pathway enrichment analysis. The larger the circle, the greater the proportion of the gene in the Gene Ontology/Kyoto Encyclopedia of Genes and Genomes. GNGT2: G protein subunit gamma transducin 2; MAPK: Mitogen-activated protein kinase; ERBB: Epidermal growth factor.

of the *SST* promoter is common and is associated with early tumor progression in Barrett's esophagus. The *SH3GL2* gene is up-regulated. The gene is not only the common DEGs of the four-stage time series but also the Hub gene in module 6. It also may play an important role in the regulation of esophageal cancer by methylation modification. Ghosh *et al*<sup>[32]</sup> studied the effect of *SH3GL2* methylation on the pathogenesis of head and neck squamous cell carcinoma, and abnormal *SH3GL2* is an independent pathway for early developmental abnormalities of the head and neck.



**Figure 3** Molecular mechanism and expression of G protein subunit gamma transducin 2 in esophageal cancer. A and B: The expression of G protein subunit gamma transducin 2 (GNGT2) in esophageal cancer patients and cell lines; C: Transfected of EC109 and KYSE70 cells; D: GNGT2 promote the proliferation of esophageal cancer cells.

## ARTICLE HIGHLIGHTS

### Research background

Esophageal cancer is one of the most lethal malignant tumors in the world. In the past decades, although the treatment methods for esophageal cancer have improved, the overall efficacy is still poor.

### Research motivation

In-depth analysis of molecular mechanisms related to esophageal cancer.

### Research objectives

Exploring the molecular process of esophageal cancer comprehensively and deeply.

### Research methods

This study used differential expression analysis, enrichment analysis, methylation analysis, survival analysis, and statistical analyses.

### Research results

A total of 7457 differentially expressed genes and 14 gene interaction modules were identified. These module genes were significantly involved in the positive regulation of protein transport, gastric acid secretion, insulin-like growth factor receptor binding and other biological processes as well as p53 signaling pathway, epidermal growth factor signaling pathway and epidermal growth factor receptor signaling pathway. In addition, transcription factors (including HIF1A) and ncRNAs (including CRNDE and hsa-mir-330-3p) that significantly regulate dysfunction modules were identified. Further, survival analysis showed that GNGT2 was closely related to survival of esophageal cancer. Differentially expressed genes with strong methylation regulation ability were identified, including *SST* and *SH3GL2*.

### Research conclusions

Overall, our work describes in detail the role of multifactor-mediated dysfunction module in the whole process of esophageal cancer, identifying key genes for staging and related biological processes, which may help to identify potential molecular mechanisms and therapeutic targets for the deterioration of esophageal cancer.

### Research perspectives

This work not only helps us to reveal the potential regulatory factors involved in the development of disease but also deepen our understanding of its deterioration mechanism.

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## Case Control Study

# Diagnostic and prognostic value of lncRNA cancer susceptibility candidate 9 in hepatocellular carcinoma

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## Abstract

### BACKGROUND

Hepatocellular carcinoma (HCC) is a common malignant gastrointestinal tumor. There are currently few clinical diagnostic and prognostic markers for HCC. lncRNA cancer susceptibility candidate 9 (CASC9) is a long-chain non-coding RNA discovered in recent years, and previous studies have found that lncRNA CASC9 participates in the occurrence and development of HCC, but its clinical value remains unclear.

### AIM

To determine the expression of lncRNA CASC9 in HCC and its diagnostic and prognostic value.

### METHODS

Data on CASC9 expression in patients with HCC were collected from the Cancer Genome Atlas (TCGA) database to analyze the relationship between CASC9 and patient survival. A total of 80 HCC patients treated in The First Affiliated Hospital of Guangxi Medical University from May 2012 to January 2014 were enrolled in the patient group, and 50 healthy subjects were enrolled in the control group during the same period. CASC9 expression in the two groups was determined using quantitative real-time polymerase chain reaction, and its diagnostic and prognostic value was analyzed based on the CASC9 data and pathological data in these HCC patients. The relationship between CASC9 and patient survival was assessed during the 5-year follow-up period.

### RESULTS

Analysis of data from TCGA database revealed that control samples showed significantly lower CASC9 expression than carcinoma tissue samples ( $P < 0.001$ ); the low CASC9 expression group had a higher survival rate than the high CASC9 expression group ( $P = 0.011$ ), and the patient group showed significantly increased expression of serum CASC9, with the area under the curve (AUC) of 0.933. CASC9 expression was related to tumor size, combined hepatitis, tumor,

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node, metastasis (TNM) staging, lymph node metastasis, differentiation and alpha fetoprotein, and the high CASC9 expression group showed lower 1-year, 3-year and 5-year survival rates than the low CASC9 expression group (all  $P < 0.05$ ). Multivariate Cox regression analysis revealed that TNM staging, lymph node metastasis, differentiation, alpha fetoprotein and CASC9 were independent factors affecting the prognosis of patients. Stage I+II patients with lymph node metastasis, low differentiation, and alpha fetoprotein  $> 200$  ng/mL had a poor 5-year survival rate.

## CONCLUSION

High CASC9 expression is beneficial in the prognosis of HCC patients. CASC9 is expected to be a potential diagnostic and prognostic indicator of HCC.

**Key words:** lncRNA cancer susceptibility candidate 9; Hepatocellular carcinoma; Prognosis; Kyoto Encyclopedia of Genes and Genomes; Gene Ontology; Competing endogenous RNA

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**Core tip:** Previous studies have found that lncRNA cancer susceptibility candidate 9 (CASC9) can promote the survival of hepatocellular carcinoma (HCC) cells through AKT ions, but it is not clear whether lncRNA CASC9 can be used as a clinical indicator of diagnosis and prognosis in patients with HCC. This study confirmed that lncRNA CASC9 can be used as a potential prognostic and diagnostic indicator in patients with HCC and it may be a potential therapeutic target for HCC.

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## INTRODUCTION

Liver cancer is a common digestive tract cancer<sup>[1]</sup>. An epidemiological investigation reported 800 thousand new and dead liver cancer patients in 2018, and liver cancer was ranked the sixth most common cancer worldwide and the second major cause of cancer death in humans<sup>[2]</sup>. Hepatocellular carcinoma (HCC), the most common liver cancer, is accompanied by insidious clinical manifestations in the early stage; thus, most HCC patients are already in middle or advanced stages when admitted to hospital, and are unsuitable for surgical treatment, which seriously affects patients' quality of life<sup>[3]</sup>. At present, there are no good diagnostic markers for early HCC, and alpha fetoprotein (AFP) is the most widely used serological marker for HCC diagnosis; however, 30% of patients with HCC do not show increased AFP, and can even be negative, which increases the diagnosis difficulty of HCC<sup>[4]</sup>. Therefore, it is particularly important to identify a biomarker of HCC with high specificity.

Due to its prominent use in various disciplines, lncRNA has been a hot research topic in recent years<sup>[5]</sup>. lncRNAs are non-coding RNAs with lengths exceeding 200 nucleotides. Initially, scholars considered that lncRNAs lacked prominent protein coding ability, but recent studies found that lncRNAs have regulatory functions in a variety of mechanisms, including epigenetic modification, transcriptional regulation and post-transcriptional modification<sup>[6-8]</sup>. A previous study demonstrated that lncRNA ZEB1-AS1 is a potential prognostic indicator of HCC due to its differential expression in this tumor<sup>[9]</sup>. Another study found that lncRNA MALAT1 promotes the proliferation, migration and invasion of HCC by antagonizing miR-142-3p<sup>[10]</sup>.

Cancer susceptibility candidate 9 (CASC9), located on human chromosome 8q21.13, is a member of the lncRNA family<sup>[11]</sup>. Previous studies revealed that CASC9 was differentially expressed in esophageal cancer and lung adenocarcinoma, and was closely related to biological functions such as proliferation, invasion and metastasis<sup>[12,13]</sup>. However, there are few studies on whether CASC9 is expressed in HCC and whether it has clinical diagnostic and prognostic value. Therefore, this

study analyzed CASC9 expression in HCC based on the Cancer Genome Atlas (TCGA) database and clinical verification, in order to provide a potential index for clinical diagnosis.

## MATERIALS AND METHODS

### **Acquisition and analysis of TCGA data**

Data on gene expression in HCC were downloaded for analysis by logging into TCGA, selecting Access TCGA Data and entering the database. The data were acquired from the core sample database of TCGA, and sequencing and analysis were performed on the acquired data based on a standardized processing scheme (<http://can-cergenome.nih.gov/cancerogenomics/tissuesamples>). The data included a total of 424 patients, involving 374 cancer samples and 50 control samples. Clinical data on HCC patients were acquired from <http://gdac.broadinstitute.org> for analysis, and excluded patients without detailed data and a survival time less than 30 d. Original data on CASC9 were processed through log (X+1, 2) to analyze the differences between control samples and carcinoma tissues, and a survival curve for high and low expression groups was drawn according to the median value of CASC9.

### **Bioinformatic analysis of lncRNA**

Starbase 3.0 was adopted to predict lncRNA CASC9 targeted microRNA (miR), and miRDB, miRTarBase, and TargetScan were adopted to predict target genes of potential miR on-line. Cytoscape software was adopted to draw the competing endogenous (ce)RNA network, and David software to analyze target genes based on the Kyoto Encyclopedia of Genes and Genomes and Gene Ontology (GO) enrichment analysis.

### **Collection of patient samples**

A total of 80 HCC patients (50 males and 30 females with an average age of  $54.6 \pm 5.0$  years) treated in The First Affiliated Hospital of Guangxi Medical University from May 2012 to January 2014 were enrolled in the patient group, and 50 healthy subjects (29 males and 21 females with an average age of  $53.7 \pm 4.1$  years) were enrolled in the control group. This study was approved by the First Affiliated Hospital of Guangxi Medical University Ethics Committee. The patients meeting the following criteria were included: Patients diagnosed with HCC based on imaging and pathologic biopsy; patients meeting the TNM staging criteria for HCC by the American Joint Committee on Cancer in 2009<sup>[14]</sup>; patients who had not taken part in any previous targeted tumor research (surgery, radiotherapy and chemotherapy, *etc.*), understood the study and signed an informed consent form (including their families) and patients whose expected survival time was more than 3 mo. The following patients were excluded: Patients with other combined tumors, renal function diseases, infection before admission, and those unwilling to cooperate.

### **Sample collection and detection**

Peripheral venous blood (5 mL) was obtained from the patients, centrifuged at 3000 rpm for 10 min after 30 min and the supernatant collected. Total RNA in the collected supernatant was extracted with TRIzol reagent (Carlsbad Invitrogen Company, California, United States), and the purity, concentration and integrity of the total RNA were determined using ultraviolet spectrophotometry and agarose gel electrophoresis. Reverse transcription was performed using TransScript® miRNA RT Enzyme Mix and 2×TS miRNA Reaction Mix in the TransScript Green Two-Step quantitative real-time polymerase chain reaction SuperMix kit (Beijing TransGen Biotech Co., Ltd., China) in strict accordance with the original kit instructions. The amplification system of CASC9 consisted of 1 µL of cDNA, 0.4 µL of upstream and downstream primers, respectively, 10 µL of 2X TransScript® Tip Green qPCR SuperMix, Passive Reference Dye (50X), and Nuclease-free water (added to 20 µL in total). The amplification conditions were as follows: Pre-denaturation at 94°C for 30 s, denaturation at 94°C for 5 s and annealing extension at 60°C for 30 s, with 40 cycles in total. Triplicate wells were prepared for each sample, and the experiment was repeated three times. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference, and 2<sup>-Δct</sup> was used to analyze the data. Experiments were carried out using a 7500 PCR instrument (ABI, United States).

### **Follow-up of patients**

The patients were followed-up for 5 years by telephone interview and clinic reexamination. During the 1<sup>st</sup> year, the patients were followed-up at the 3<sup>rd</sup>, 6<sup>th</sup>, 9<sup>th</sup> and 12<sup>th</sup> month, respectively, and during the remaining 4 years, they were followed-up

once every 4 mo.

### Statistical analysis

In this study, the acquired data were statistically analyzed using the SPSS20.0 software package, and figures were drawn using the GraphPad 7 software package. The distribution of measurement data was analyzed using the *K-S* test, and the data in normal distribution were expressed as mean  $\pm$  SD. Comparisons between groups were performed using independent-samples *T* test. Comparisons within groups were analyzed by paired *t* test, and expressed as *t*. Ranked data were analyzed using the rank sum test, and expressed as *Z*. Enumeration data were analyzed using the chi-square test. ROC curves of CASC9 to evaluate its diagnostic value in HCC were drawn, and a K-M curve for 5-year survival of patients was also drawn. The log-rank test was adopted for analysis. Multivariate Cox regression analysis was adopted to analyze independent risk factors for patients. *P* < 0.05 indicated a significant difference.

## RESULTS

### CASC9 expression in TCGA

Analysis of data from TCGA database on CASC9 expression in HCC patients showed that control samples had significantly lower CASC9 expression than carcinoma tissue samples (*P* < 0.001), and the grouping of patients according to median CASC9 expression revealed that the low CASC9 expression group had a higher survival rate than the high CASC9 expression group (*P* = 0.011, Figure 1).

### Comparison of baseline data

Comparisons between the patient group and control group showed that there were no significant differences between the groups in terms of gender, age, body mass index (BMI), past medical history, smoking history, history of alcoholism and place of residence, while a difference in AFP expression was observed between the two groups (*P* < 0.001, Table 1).

### CASC9 expression in patients and its clinical value

The expression of serum CASC9 in the patient group was significantly higher than that in the control group, and the ROC curve showed that the AUC was 0.933. Further analysis of the relationship between CASC9 and clinical pathological data showed that tumor size, combined hepatitis, TNM staging, lymph node metastasis, differentiation and AFP were closely related to CASC9 expression. In addition, the analysis of ROC curves showed that CASC9 was associated with tumor size, TNM staging, lymph node metastasis, differentiation and AFP (Figure 2 and Tables 2 and 3).

### Relationship between CASC9 and patients' survival

All the patients were successfully followed-up for 5 years in terms of survival. The patient group showed a 5-year survival rate of 17.50% with 14 patients surviving. The patients were grouped into the high CASC9 expression group and the low CASC9 expression group according to the median CASC9 expression (3.305), and survival was compared. The high CASC9 expression group showed significantly lower 1-year, 3-year and 5-year survival rates than the low CASC9 expression group (all *P* < 0.05, Figure 3).

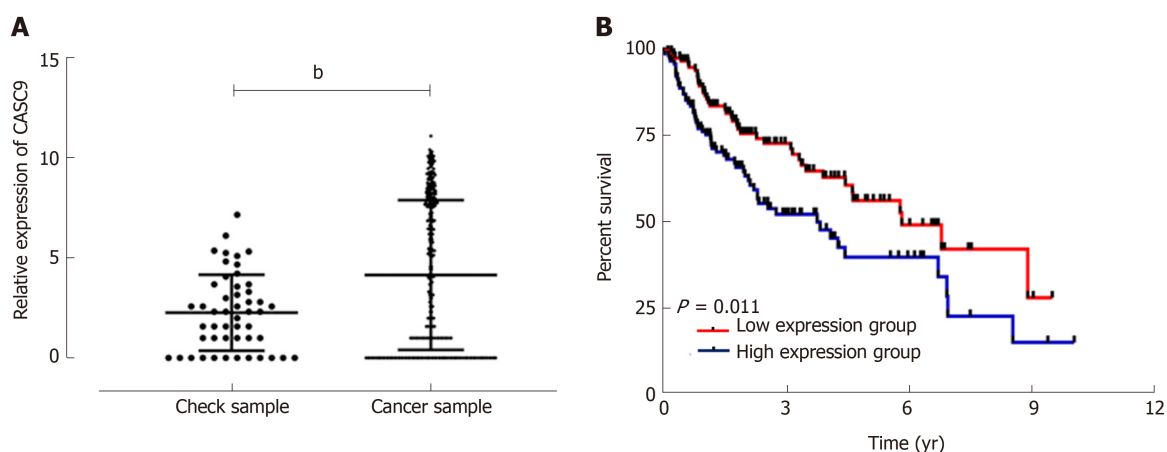
### Independent factors affecting prognosis of patients

Univariate Cox regression analysis of the pathological data showed that TNM staging, lymph node metastasis, differentiation, AFP and CASC9 were factors which affected the prognosis of patients, and multivariate Cox regression analysis of these factors showed that TNM staging, lymph node metastasis, differentiation, AFP, and CASC9 were independent factors affecting the prognosis of patients (Table 4). In addition, the survival curves in terms of TNM staging, lymph node metastasis, differentiation, AFP and patients' 5-year survival indicated that stage I+II patients with lymph node metastasis, low differentiation, and AFP > 200 ng/mL showed poor 5-year survival (Figure 4).

### Bioinformatic analysis

Seventeen CASC9 potential targeted miRs were identified by Starbase 3.0, and 89 mRNAs were found by predicting the downstream mRNA of the 17 targeted miRs with miRDB, miRTarBase, and TargetScan. We used Cytoscape software to construct the interaction map between lncRNA-miRNA-mRNAs, and GO enrichment and





**Figure 1** Cancer susceptibility candidate 9 expression and survival from TCGA database. A: Cancer susceptibility candidate 9 (CASC9) was highly expressed in cancer patients; B: The survival rate of the high CASC9 expression group was lower than that of the low CASC9 expression group ( $P = 0.011$ ). <sup>b</sup> $P < 0.01$ . CASC9: Cancer susceptibility candidate 9.

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis were performed on the 89 mRNAs in the ceRNA network using DAVID and KOBAS, and 18 GO functions with  $P < 0.05$  and 8 signal transduction pathways with  $P < 0.05$  were found (Tables 5 and 6).

## DISCUSSION

HCC, a digestive system neoplasm with high incidence and mortality, is a common disease in male patients, and morbidity in men compared to women is 2.81:1<sup>[15]</sup>. A Chinese tumor epidemiology survey reported that more than 400 thousand new and dead patients with HCC were observed in 2015; however, there is no effective diagnosis and treatment plan for this disease<sup>[16]</sup>. At present, AFP is the main clinical serodiagnostic index for HCC, but relevant studies have found that AFP expression increases in liver diseases such as hepatitis; thus, its specificity is low<sup>[17]</sup>. Therefore, the key to resolving the problem is to identify biological indices with high sensitivity and specificity.

LncRNA is a long-chain non-coding RNA. A previous study demonstrated that lncRNAs participate in the occurrence and development of various cancers<sup>[18]</sup>. A study by Ma *et al*<sup>[19]</sup> found that the biological function of HCC was inhibited by regulating the expression of miR-122-5p in HCC based on lncRNA ANRIL knockout. CASC9 is a newly discovered tumor susceptibility gene. A study by Luo *et al*<sup>[20]</sup> found that CASC9 and CPSF3 could cooperatively regulate TGF- $\beta$  signaling pathway conduction in colorectal cancer, and a study by Liang *et al*<sup>[21]</sup> reported that CASC9 promoted metastasis of esophageal squamous cell carcinoma by up regulating LAMC2 expression through an interaction with CREB binding protein. However, there are few reports on CASC9 in HCC at present. A study by Noh *et al*<sup>[22]</sup> showed that CASC9-mediated AKT ions promoted the survival of HCC cells, and there are no other relevant studies on whether CASC9 can be used as a diagnostic and prognostic indicator of HCC. Therefore, this study confirmed the expression and prognostic value of CASC9 in HCC based on the TCGA database, in order to provide a new potential clinical index.

TCGA database, one of the largest Cancer Genome Projects, contains a variety of tumor gene data<sup>[23]</sup>. In this study, we first extracted data on CASC9 expression in the tissues of HCC patients from TCGA database, analyzed these tissues, and found that CASC9 expression in carcinoma tissue samples was significantly higher than that in adjacent control tissue samples. The patients were divided into the high CASC9 expression group and the low CASC9 expression group according to the median CASC9 expression to determine survival of the patients. It was found that the low CASC9 expression group had a higher survival rate than the high CASC9 expression group. The above results indicated that CASC9 may be a potential diagnostic and prognostic indicator of HCC. Further clinical research demonstrated that the expression of serum CASC9 in HCC patients was consistent with the data from TCGA database. In addition, we found that the AUC of CASC9 expression ( $> 0.9$ ) had very high diagnostic value based on ROC curves. We further analyzed the relationship between CASC9 and 1-year, 3-year and 5-year survival rates in HCC patients, and



Table 1 Baseline analysis, *n* (%)

Factors		Patient group ( <i>n</i> = 80)	Control group ( <i>n</i> = 50)	$\chi^2$ / <i>t</i>	<i>P</i> value
Gender				0.264	0.609
	Male	50 (62.50)	29 (58.00)		
	Female	30 (37.50)	21 (42.00)		
Age (yr)		54.6 ± 5.0	53.7 ± 4.1	1.068	0.288
BMI (kg/m <sup>2</sup> )		22.86 ± 1.93	23.17 ± 2.07	0.866	0.388
Past medical history					
	Hypertension	25 (31.25)	10 (20.00)	1.979	0.160
	Hyperlipidemia	13 (16.25)	6 (12.00)	0.445	0.505
	Diabetes	20 (25.00)	10 (20.00)	0.433	0.510
Smoking history				0.081	0.776
	Yes	50 (62.50)	30 (60.00)		
	No	30 (37.50)	20 (40.00)		
History of alcoholism				0.494	0.482
	Yes	15 (18.75)	7 (14.00)		
	No	65 (81.25)	43 (86.00)		
Place of residence				0.177	0.674
	Urban area	45 (56.25)	30 (60.00)		
	Rural area	35 (43.75)	20 (40.00)		
Tumor size					
	≥ 5 cm	45 (56.25)			
	< 5 cm	35 (43.75)			
Combined hepatitis					
	Yes	70 (87.50)			
	No	10 (12.50)			
TNM staging					
	Stage I+II	33 (41.25)			
	Stage III+IV	47 (58.75)			
Lymph node metastasis					
	Yes	42 (52.50)			
	No	38 (47.50)			
Differentiation					
	Low differentiation	27 (33.75)			
	Moderate + high differentiation	53 (66.25)			
AFP (ng/mL)				59.583	< 0.001
	≤ 200	25 (31.25)	50 (100.00)		
	>200	55 (68.75)	0 (0.00)		

BMI: Body mass index; AFP: Alpha fetoprotein; TNM: Tumor, node, metastasis.

differences between the low CASC9 expression group and high CASC9 expression group in 1-year, 3-year and 5-year survival rates were found, which indicated that CASC9 could be adopted as an index for determining the short-term and long-term survival rates of HCC patients. We also analyzed the relationship between CASC9 and pathological data, and found that CASC9 was closely related to tumor size, TNM staging, lymph node metastasis, differentiation, and AFP, which indicated that CASC9 was closely related to the occurrence and development of HCC. In addition, CASC9 had certain diagnostic value in determining tumor size, TNM staging, lymph node metastasis, differentiation and AFP. A study by Gao *et al*<sup>[24]</sup> revealed that CASC9 was also highly expressed in esophageal carcinoma, and had relatively high diagnostic value (AUC: 0.814). However, the AUC of CASC9 in our study group was 0.933. This indicated that CASC9 may have higher diagnostic value for HCC than for esophageal carcinoma, but whether it is a diagnostic index still requires further investigation.

Pathological data on HCC patients were obtained to further analyze independent factors affecting the prognosis of patients, and TNM staging, lymph node metastasis,

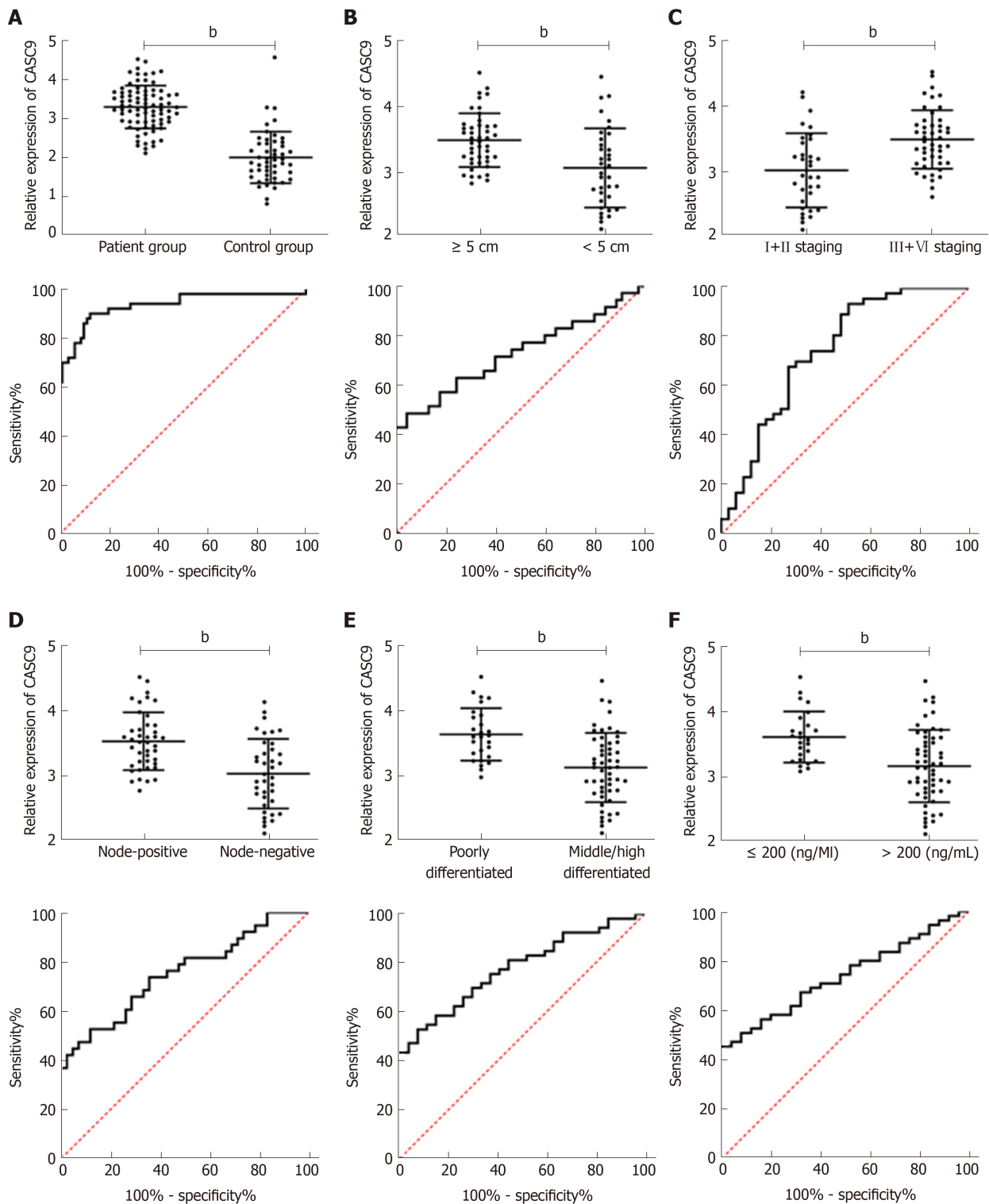
**Table 2 Relationship between cancer susceptibility candidate 9 and pathological data**

Factors	Patient group (n = 80)	T	P value
Gender		0.308	0.759
	Male (n = 50)	3.287 ± 0.553	
	Female (n = 30)	3.326 ± 0.553	
Age (yr)		0.751	0.455
	< 55 (n = 35)	3.354 ± 0.504	
	≥ 55 (n = 45)	3.261 ± 0.585	
Tumor size		4.357	< 0.001
	≥ 5 cm (n = 45)	3.489 ± 0.414	
	< 5 cm (n = 35)	3.06 ± 0.611	
Combined hepatitis		0.575	0.567
	Yes (n = 70)	3.315 ± 0.540	
	No (n = 10)	3.208 ± 0.634	
TNM stage		4.157	< 0.001
	Stage I+II (n = 33)	3.496 ± 0.448	
	Stage III+IV (n = 47)	3.024 ± 0.567	
Lymph node metastasis		4.557	< 0.001
	Yes (n = 42)	3.54 ± 0.445	
	No (n = 38)	3.038 ± 0.538	
Differentiation		4.387	< 0.001
	Low differentiation (n = 27)	3.642 ± 0.405	
	Moderate + high differentiation (n = 53)	3.128 ± 0.535	
AFP (ng/mL)		3.617	0.001
	≤ 200 (n = 25)	3.609 ± 0.393	
	> 200 (n = 55)	3.162 ± 0.557	

AFP: Alpha fetoprotein; TNM: Tumor, node, metastasis.

differentiation, AFP and CASC9 were found to be independent prognostic factors for HCC patients based on multivariate analysis. A study by Tan and Huang confirmed that TNM staging, lymph node metastasis, differentiation, and AFP were closely related to the prognosis of patients, which was consistent with the findings in our study<sup>[25,26]</sup>. However, we found that tumor size was not related to prognosis in this study, which was inconsistent with the previous study findings. We speculated that this may be related to the collected samples. The tumor size data in this study were relatively intensively distributed with an average size of  $4.8 \pm 0.8$  cm, which may be the reason for this difference. Our study confirmed, for the first time, that CASC9 was an independent prognostic factor for HCC patients, indicating that CASC9 may be a prognostic and diagnostic index for HCC.

A co-expression network of lncRNA-miR-mRNA was constructed and 17 CASC9 targeted miRs, and 89 targeted mRNAs were observed. The top 10 functions in GO enrichment analysis were negative regulation of transcription from the RNA polymerase II promoter, positive regulation of transcription regulatory region DNA binding, cytoplasm, positive regulation of transcription, DNA-templated, osteoblast differentiation, neuron projection morphogenesis, transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific, negative regulation of sequence-specific DNA binding transcription factor activity, and neuron projection, respectively, and the 8 different signal transduction pathways in the Kyoto Encyclopedia of Genes and Genomes analysis were microRNAs in cancer, pathways in cancer, p53 signaling pathway, Chagas disease (American trypanosomiasis), proteoglycans in cancer, cell cycle, PI3K-Akt signaling pathway, and Hippo signaling pathway, respectively. According to the above research, CASC9 can participate in various biological pathways by regulating the miR/mRNA axis, and it is noteworthy that signal pathways such as microRNAs in cancer, pathways in cancer, p53 signaling pathway, PI3K-Akt signaling pathway, and Hippo signaling pathway are important cancer-related signal transduction pathways<sup>[27-29]</sup>. CASC9 can also participate in the occurrence of these pathways by regulating the miR/mRNA axis, which provides an important cornerstone for our subsequent research.



**Figure 2** Cancer susceptibility candidate 9 expression in patients, its relationship with tumor size, Tumor, Node, Metastasis staging, lymph node metastasis, differentiation, and alpha fetoprotein expression, and its diagnostic value. A: Cancer susceptibility candidate 9 (CASC9) was highly expressed in the patient group, and the AUC was 0.933; B: Relationship between CASC9 expression and tumor size. The AUC was 0.726; C: Relationship between CASC9 expression and TNM staging. The AUC was 0.743; D: Relationship between CASC9 expression and lymph node metastasis. The AUC was 0.752; E: Relationship between CASC9 expression and differentiation. The AUC was 0.777; F: Relationship between CASC9 expression and AFP. The AUC was 0.738. <sup>b</sup>*P* < 0.01. CASC9: Cancer susceptibility candidate 9; AUC: area under the curve.

Although this study confirmed the value of CASC9 in HCC, it still has some limitations. Firstly, we only analyzed patients with HCC and healthy subjects, but did not determine CASC9 expression in patients with hepatitis; thus, whether CASC9 can distinguish HCC from hepatitis still requires further research in healthy subjects. Secondly, how CASC9 participates in the occurrence and development of HCC is unclear. Lastly, this study only focused on Chinese patients, and whether CASC9

**Table 3** Receiver operating characteristic parameters

Factors	HCC diagnosis	Tumor size	TNM stage	Lymph node metastasis	Differentiation	AFP
AUC	0.933	0.726	0.743	0.752	0.777	0.738
SD	0.026	0.060	0.059	0.055	0.051	0.054
95%CI	0.882-0.983	0.608-0.845	0.628-0.858	0.645-0.859	0.678-0.876	0.632-0.844
<i>P</i> value	< 0.001	0.001	< 0.001	< 0.001	< 0.001	0.001
Specificity	87.50%	95.56%	48.48%	88.10%	92.59%	100.00%
Sensitivity	90.00%	48.57%	93.62%	52.63%	52.83%	45.45%
Youden index	77.50%	44.13%	42.10%	40.73%	45.42%	45.45%
Cut-off	< 2.672	< 2.916	> 2.919	< 3.057	< 3.148	< 3.057

AUC: Area under curve; HCC: Hepatocellular carcinoma; AFP: Alpha fetoprotein.

expression is increased in HCC patients of different races is unclear. Therefore, we hope to carry out bioinformatics and a basic study in the future to further analyze the way that CASC9 affects the occurrence and development of HCC, and obtain different pathological specimens for CASC9 detection in difference races, in order to address the shortcomings in this study. In conclusion, high CASC9 expression is beneficial for the prognosis of HCC patients, and CASC9 is expected to be a potential diagnostic and prognostic indicator of HCC.

**Table 4 Multivariate Cox regression analysis**

Factors	Univariate Cox			Multivariate Cox		
	P value	HR	95%CI	P value	HR	95%CI
Gender (male <i>vs</i> female)	0.272	1.320	1.320-0.804			
Age (< 55 yr <i>vs</i> ≥ 55 yr)	0.438	0.825	0.825-0.508			
Tumor size (≥ 5 cm <i>vs</i> < 5 cm)	0.952	1.015	1.015-0.624			
Combined hepatitis (yes <i>vs</i> no)	0.636	1.185	1.185-0.586			
TNM staging (stage I+II <i>vs</i> stage III+IV)	0.000	4.271	4.271-2.391	0.006	2.501	1.308-4.781
Lymph node metastasis (yes <i>vs</i> no)	0.001	0.428	0.428-0.259	0.025	0.535	0.309-0.924
Differentiation (low <i>vs</i> medium + high)	0.000	0.242	0.242-0.144	0.000	0.326	0.186-0.569
AFP (≤ 200 <i>vs</i> > 200 ng/mL)	0.025	1.914	1.914-1.086	0.042	1.935	1.023-3.662
CASC9 (< 3.305 <i>vs</i> ≥ 3.305)	0.005	2.023	2.023-1.235	0.021	2.024	1.112-3.682

AFP: Alpha fetoprotein; TNM: Tumor, node, metastasis; CASC9: Cancer susceptibility candidate 9.

**Table 5 The top 10 Gene Ontology enrichment functions**

Term	Count	P value	Genes
Negative regulation of transcription from the RNA polymerase II promoter	9	0.001	PHF19, SQSTM1, CPEB3, E2F7, ESR1, CBX2, SOX6, HMGA2, TWIST1
Positive regulation of transcription regulatory region DNA binding	3	0.002	WNT3A, HMGA2, TWIST1
Cytoplasm	27	0.003	CLSPN, CPEB3, TPM2, BDNF, RNF165, MAPT, STRIP2, HOXA10, FASN, PLCB1, CDC37L1, ARL2, IRAK1, SGK1, MAP2K1, KIF5A, DDX39B, PIM1, SOCS6, ESR1, SNAI2, WEE1, CDC25A, ADM, CA8, PSAT1, DUSP6
Positive regulation of transcription, DNA-templated	6	0.009	RET, MAP2K1, FOXK1, MYRF, ESR1, PLCB1
Osteoblast differentiation	4	0.010	WNT3A, FASN, SNAI2, TWIST1
Neuron projection morphogenesis	3	0.011	BDNF, SGK1, WEE1
Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific	5	0.019	PLAG1, HOXA10, ESR1, HMGA2, MYBL2
Negative regulation of sequence-specific DNA binding transcription factor activity	3	0.021	PIM1, ESR1, TWIST1
Neuron projection	4	0.027	CPEB3, KIF5A, MAPT, SLC6A4

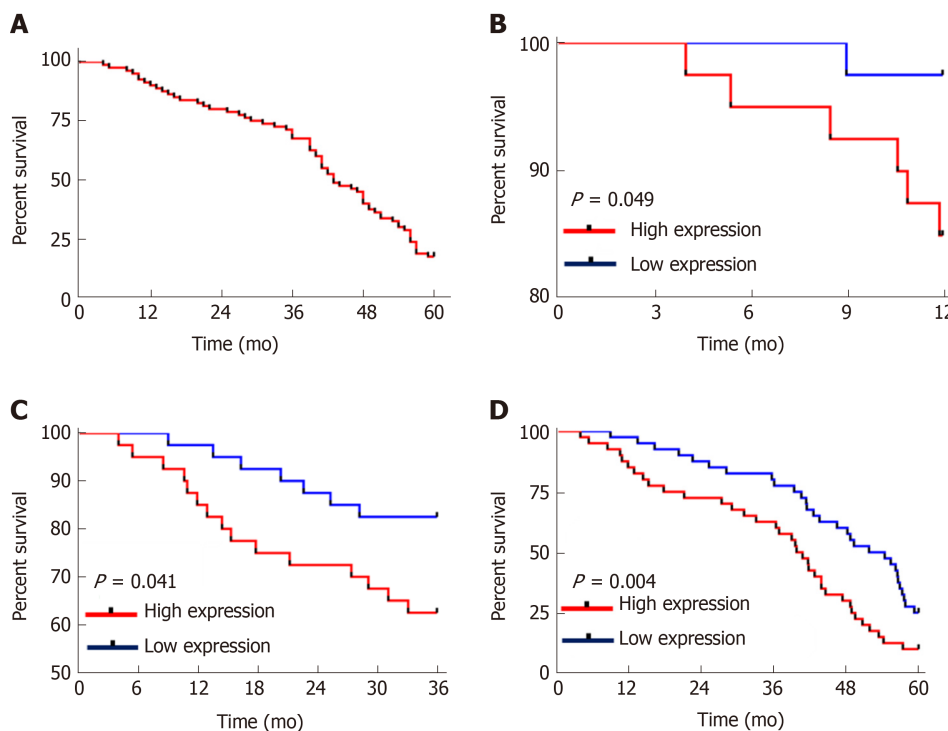
PHF19: PHD finger protein 19; SQSTM1: Sequestosome 1; CPEB3: Cytoplasmic polyadenylation element binding protein 3; E2F7: E2F transcription factor 7; ESR1: Estrogen receptor 1; CBX2: Chromobox 2; SOX6: SRY-box transcription factor 6; HMGA2: High mobility group AT-hook 2; TWIST1: Twist family bHLH transcription factor 1; WNT3A: Wnt family member 3A; CLSPN: Claspin; TPM2: Tropomyosin 2; BDNF: Brain derived neurotrophic factor; RNF165: Ring finger protein 165; MAPT: Microtubule associated protein tau; STRIP2: Striatin interacting protein 2; HOXA10: Homeobox A10; FASN: Fatty acid synthase; PLCB1: Phospholipase C beta 1; CDC37L1: Cell division cycle 37 like 1; ARL2: ADP ribosylation factor like GTPase 2; IRAK1: Interleukin 1 receptor associated kinase 1; SGK1: Serum/glucocorticoid regulated kinase 1; MAP2K1: Mitogen-activated protein kinase kinase 1; KIF5A: Kinesin family member 5A; DDX39B: DEXD-box helicase 39B; PIM1: Pim-1 proto-oncogene, serine/threonine kinase; SOCS6: Suppressor of cytokine signaling 6; SNAI2: Snail family transcriptional repressor 2; WEE1: WEE1 G2 checkpoint kinase; CDC25A: Cell division cycle 25A; ADM: Adrenomedullin; CA8: Carbonic anhydrase 8; PSAT1: Phosphoserine aminotransferase 1; DUSP6: Dual specificity phosphatase 6; RET: Ret proto-oncogene; FOXK1: Forkhead box K1; MYRF: Myelin regulatory factor; PLAG1: PLAG1 zinc finger; MYBL2: MYB proto-oncogene like 2; SLC6A4: Solute carrier family 6 member 4.



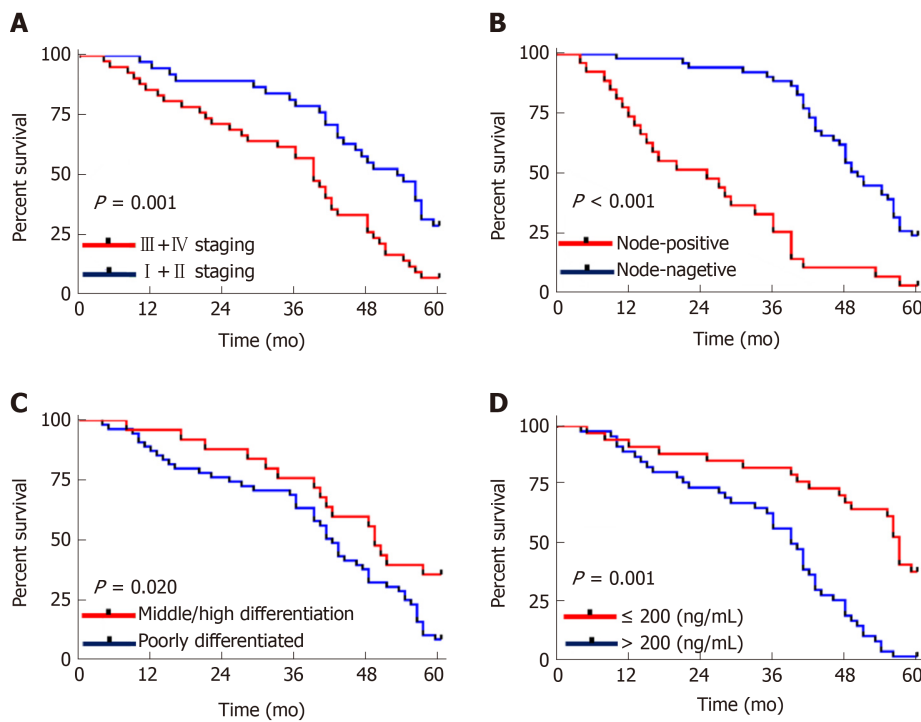
**Table 6** The top10 Kyoto Encyclopedia of Genes and Genomes signal pathways

Term	Count	P value	Genes
MicroRNAs in cancer	7	0.002	KIF23, CCNE1, MAP2K1, WNT3A, PIM1, HMGA2, CDC25A
Pathways in cancer	8	0.005	CCNE1, RET, MAP2K1, WNT3A, ITGA2, LAMC1, AXIN2, PLCB1
p53 signaling pathway	4	0.007	STEAP3, CCNE1, SERPINE1, CHEK1
Chagas disease (American trypanosomiasis)	4	0.024	GNAL, IRAK1, SERPINE1, PLCB1
Proteoglycans in cancer	5	0.024	MAP2K1, WNT3A, ESR1, ITGA2, TWIST1
Cell cycle	4	0.030	CCNE1, CHEK1, WEE1, CDC25A
PI3K-Akt signaling pathway	6	0.038	CCNE1, SGK1, MAP2K1, ITGA2, LAMC1, ANGPT2
Hippo signaling pathway	4	0.048	WNT3A, SERPINE1, AXIN2, SNAI2
Small cell lung cancer	3	0.081	CCNE1, ITGA2, LAMC1
Progesterone-mediated oocyte maturation	3	0.082	MAP2K1, CPEB3, CDC25A

KIF23: Kinesin family member 23; CCNE1: Cyclin E1; MAP2K1: Mitogen-activated protein kinase kinase 1; WNT3A: Wnt family member 3A; PIM1: Pim-1 proto-oncogene, serine/threonine kinase; HMGA2: High mobility group AT-hook 2; CDC25A: Cell division cycle 25A; RET: Ret proto-oncogene; ITGA2: Integrin subunit alpha 2; LAMC1: Laminin subunit gamma 1; AXIN2: Axin 2; PLCB1: Phospholipase C beta 1; STEAP3: STEAP3 metalloredutase; SERPINE1: Serpin family E member 1; CHEK1: Checkpoint kinase 1; CPEB3: Cytoplasmic polyadenylation element binding protein 3; SGK1: Serum/glucocorticoid regulated kinase 1; ANGPT2: Angiopoietin 2; WEE1: WEE1 G2 checkpoint kinase; ESR1: Estrogen receptor 1; TWIST1: Twist family bHLH transcription factor 1; GNAL: G protein subunit alpha L; IRAK1: Interleukin 1 receptor associated kinase 1; SNAI2: Snail family transcriptional repressor 2.



**Figure 3** Relationship between cancer susceptibility candidate 9 and patients' survival. A: The overall survival of patients; B: The 1-year survival of patients in the high and low Cancer susceptibility candidate 9 (CASC9) expression groups ( $P = 0.049$ ); C: The 3-year survival of patients in the high and low CASC9 expression groups ( $P = 0.041$ ); D: The 5-year survival of patients in the high and low CASC9 expression groups ( $P = 0.004$ ). CASC9: Cancer susceptibility candidate 9.



**Figure 4 Relationship between tumor, node, metastasis staging, lymph node metastasis, differentiation, alpha fetoprotein and 5-year survival of patients.** A: The 5-year survival rate of stage I+II patients was higher than that of stage III+IV patients ( $P = 0.001$ ); B: The 5-year survival rate of patients without lymph node metastasis was higher than that of patients with lymph node metastasis ( $P < 0.001$ ); C: The 5-year survival rate of patients with moderate/high differentiation was higher than that of patients with low differentiation ( $P = 0.020$ ); D: The 5-year survival rate of patients with AFP  $\leq 200$  was higher than that of patients with AFP  $> 200$  ( $P = 0.001$ ).

## ARTICLE HIGHLIGHTS

### Research background

Liver cancer, the sixth most common cancer worldwide, is the second leading cause of cancer mortality. A lncRNA is a non-coding RNA with a length exceeding 200 nucleotides. Previous studies have found that lncRNAs are involved in the development and progression of hepatocellular carcinoma (HCC), but whether they can be used as potential diagnostic and prognostic indicators is unclear.

### Research motivation

lncRNA is a newly discovered non-coding RNA. Some studies have revealed that lncRNAs are differentially expressed in HCC and are expected to be measures of potential outcome. In this study, we found that lncRNA CASC9 was highly expressed in HCC patients based on the Cancer Genome Atlas (TCGA) database analysis and patients with high expression of CASC9 had a poor prognosis, which indicated that lncRNA CASC9 may be a potential diagnostic and prognostic indicator of HCC.

### Research objectives

This study aimed to identify the expression of lncRNA CASC9 in HCC, its diagnostic and prognostic value and to construct ceRNA network maps to further explore its underlying mechanism.

### Research methods

Data on the expression of lncRNA CASC9 in TCGA were extracted, clinical samples were collected to further determine the expression of lncRNA CASC9 in HCC, and the correlation between lncRNA CASC9 and pathological data and survival of HCC patients were analyzed. Potential microRNA and target genes of lncRNA CASC9 were analyzed using on-line prediction websites, and ceRNA maps were drawn. In addition, Kyoto Encyclopedia of Genes and Genomes and GO enrichment analysis were employed to analyze the potential mechanisms of lncRNA CASC9 in biological processes.

### Research results

Analysis revealed that lncRNA CASC9 was highly expressed in the tissues and serum of HCC patients and high expression of CASC9 was related to tumor size, TNM staging, lymph node metastasis, differentiation, and AFP. Further analysis showed that lncRNA CASC9 could be used as a diagnostic indicator of the above indices. Prognostic analysis revealed that the survival rate of patients with high expression of lncRNA CASC9 decreased, and Cox regression analysis

showed that lncRNA CASC9 could be used as an independent prognostic indicator in HCC patients. The bioinformatics analysis revealed that lncRNA CASC9 potentially targeted 17 miRs. A total of 89 mRNAs were found in mRNA prediction. GO enrichment and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis revealed that lncRNA CASC9 participated in 18 GO functions and 8 signal transduction pathways.

### Research conclusion

lncRNA CASC9 is highly expressed in HCC patients, and may be a potential diagnostic and prognostic indicator of HCC.

### Research perspectives

It is necessary to further explore the value of lncRNA CASC9 in HCC, and prospective experiments and multi-center clinical studies are required to obtain more robust conclusions. In addition, we hope to further verify the relevant mechanisms of lncRNA CASC9 in HCC by carrying out relevant basic experiments, in order to address the deficiencies in this study.

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## Retrospective Study

# Operative complications and economic outcomes of cholecystectomy for acute cholecystitis

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**Author contributions:** Rice CP, Vaishnavi KB, Chao C, Jenson WR, Griffin LW and Mileski WJ contributed to the conception and design of the study; Rice CP, Vaishnavi KB, Chao C and Jenson WR assisted with data acquisition; Rice CP, Chao C, Jupiter D and Mileski WJ analyzed and interpreted the data obtained; Rice CP, Chao C, Schaeffer AB, Jupiter D and Mileski WJ drafted the article and/or made critical revisions related to important intellectual content of the manuscript; all authors contributed to the proof-reading and final approval of the version of the article to be published.

### Institutional review board

**statement:** This study was approved by the UTMB Institutional Review Board, No. 18-0042.

**Informed consent statement:** This manuscript is a retrospective study, therefore, signed informed consent forms are not necessary.

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

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## Abstract

### BACKGROUND

Recent management of acute cholecystitis favors same admission (SA) or emergent cholecystectomy based on overall shorter hospital stay and therefore cost savings. We adopted the practice of SA cholecystectomy for the treatment of acute cholecystitis at our tertiary care center and wanted to evaluate the economic benefit of this practice. We hypothesized that the existence of complications, particularly among patients with a higher degree of disease severity, during SA cholecystectomy could negate the cost savings.

### AIM

To compare complication rates and hospital costs between SA *vs* delayed cholecystectomy among patients admitted emergently for acute cholecystitis.

### METHODS

Under an IRB-approved protocol, complications and charges for were obtained for SA, later after conservative management (Delayed), or elective cholecystectomies over an 8.5-year period. Patients were identified using the acute care surgery registry and billing database. Data was retrieved *via* EMR, operative logs, and Revenue Cycle Operations. The severity of acute cholecystitis was graded according to the Tokyo Guidelines. TG18 categorizes acute cholecystitis by Grades 1, 2, and 3 representing mild, moderate, and severe, respectively. Comparisons were analyzed with  $\chi^2$ , Fisher's exact test, ANOVA, *t*-tests, and logistic regression; significance was set at  $P < 0.05$ .



interest.

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## RESULTS

Four hundred eighty-six (87.7%) underwent a SA while 68 patients (12.3%) received Delayed cholecystectomy. Complication rates were increased after SA compared to Delayed cholecystectomy (18.5% *vs* 4.4%,  $P = 0.004$ ). The complication rates of patients undergoing delayed cholecystectomy was similar to the rate for elective cholecystectomy (7.4%,  $P = 0.35$ ). Mortality rates were 0.6% *vs* 0% for SA *vs* Delayed. Patients with moderate disease (Tokyo 2) suffered more complications among SA while none who were delayed experienced a complication (16.1% *vs* 0.0%,  $P < 0.001$ ). Total hospital charges for SA cholecystectomy were increased compared to a Delayed approach (\$44500  $\pm$  \$59000 *vs* \$35300  $\pm$  \$16700,  $P = 0.019$ ). The relative risk of developing a complication was 4.2x [95% confidence interval (CI): 1.4-12.9] in the SA *vs* Delayed groups. Among eight patients (95%CI: 5.0-12.3) with acute cholecystitis undergoing SA cholecystectomy, one patient will suffer a complication.

## CONCLUSION

Patients with Tokyo Grade 2 acute cholecystitis had more complications and increased hospital charges when undergoing SA cholecystectomy. This data supports a selective approach to SA cholecystectomy for acute cholecystitis.

**Key words:** Acute cholecystitis; Tokyo guidelines; Cholecystectomy; Complications; Delayed cholecystectomy

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**Core tip:** Patients presenting with acute cholecystitis (Tokyo Grade 2) have more complications and increased hospital charges when undergoing same admission cholecystectomy. This data supports a selective approach; greater disease severity may have a lower complication rate when surgery is delayed.

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## INTRODUCTION

Recent recommendations for early or same admission (SA) cholecystectomy<sup>[1-8]</sup> have become standard practice based mainly on a shorter hospital stay and thus a presumed economic benefit<sup>[9]</sup>. However, patients who present with acute cholecystitis are a heterogeneous population and for some patients, emergent cholecystectomy may result in complications that might be mitigated if the procedure were delayed. The Tokyo Guidelines (TG) were developed to guide the treatment of acute cholecystitis and to use clinical parameters at presentation to predict risk of complications associated with disease severity. TG defines the criteria for a clinical diagnosis of acute cholecystitis and disease severity grading<sup>[10]</sup>. The guidelines, first published in 2007, have been revised in 2013 and most recently in 2018. The grading system for TG13 is the same as TG18: Mild cholecystitis (Grade 1) refers to acute cholecystitis in a healthy patient with mild inflammatory changes in the gallbladder, fever and or elevated white blood cell count (WBC), and no organ dysfunction. Moderate cholecystitis (Grade 2) is associated with complaints > 72 h, a WBC  $\geq$  18K, signs of marked local inflammation (e.g., gangrenous or emphysematous cholecystitis, pericholecystic or hepatic abscess, biliary peritonitis) or a palpable tender mass in the right upper quadrant of the abdomen. Severe cholecystitis (Grade 3) is defined by concurrent organ system dysfunction. TG13/18 severity grading has been shown to correlate with hospital length of stay, conversion to open cholecystectomy, and higher morbidity and mortality<sup>[10]</sup>.

The World Society of Emergency Surgery (WSES)<sup>[1]</sup> acknowledges that there are no validated clinical scores to guide clinicians for the evaluation of surgical risk for early operative intervention of acute calculus cholecystitis. For example, one criticism of the

TG is that either drainage or cholecystectomy can be performed for severity Grade 2<sup>[11]</sup>, without specific practice guidelines indicating which therapeutic option is better. Nonetheless, the TG grading correlates with morbidity and mortality<sup>[10,12]</sup>. Further validating the preoperative value of TG grading, Ambe *et al*<sup>[13]</sup> demonstrated that increasing severity Tokyo grades significantly correlated worse disease with histologic criteria.

Using the TG severity of the disease grading system, we compared the outcomes of patients operated on during the SA *vs* delayed. Since patients with a higher grade of disease (Grades 2 and 3) are expected to have a greater rate of complications and longer length of hospital stay (compared to Grade 1), we hypothesized that the economic benefit of early cholecystectomy in the setting of complications would be negated.

## MATERIALS AND METHODS

Under an Institutional Review Board-approved protocol, we retrospectively reviewed 2118 medical charts of all patients who underwent a cholecystectomy over an 8.5-year period (February 2010 to August 2018) at the University of Texas Medical Branch (UTMB Health) – a tertiary referral center located in Galveston, Texas. Patient information was retrieved using the UTMB Health databases for acute care surgery, billing, and Revenue Cycle Operations. Patient medical charts were reviewed directly from UTMB Health's electronic medical records (EMR, Epic Hyperspace 2017, Epic Systems Corporation). A total of 26 faculty surgeons performed the cholecystectomies during this time period.

Inclusion criteria for each acutely ill patient consisted of a diagnosis of acute cholecystitis upon presentation to any UTMB Health emergency department with admission or direct admit to the hospital. All patients underwent a cholecystectomy performed either during their index admission (*i.e.*, SA) or electively after presenting with acute disease (*i.e.*, delayed cholecystectomy). For comparison only, we also abstracted the charts of all patients who underwent an elective cholecystectomy (non-acute) for a diagnosis of sub-acute conditions (*e.g.*, chronic cholecystitis (biliary colic), cholelithiasis, biliary dyskinesia, porcelain gallbladder, or gallbladder polyp). Exclusion criteria were acutely ill patients without a diagnosis of acute cholecystitis and patients < 18 years of age. We also excluded 27 patients who had a diagnosis of acute cholecystitis but underwent preoperative cholecystostomy tube placement because these patients were deemed to be poor operative candidates initially and were temporized with a drainage procedure first.

We collected information on baseline patient characteristics including demographics, insurance status, comorbidities, diagnoses, health behaviors, dates of presentation, body temperature at presentation, hospital lengths of stay, laboratory values at presentation, complications. Outcome calculations were rates of cholecystectomy on index admission, complication rates, readmission rates, and charges.

We identified data regarding charges incurred for each case from UTMB Health Revenue Cycle Operations using Current Procedural Terminology (CPT) codes, International Classification of Diseases (ICD) codes, medical record numbers of the 1882 patients involved in the study, and the operative dates between April 2013 to August 2018 (hospital charges prior to April 2013 were not activated in our electronic medical record system). The CPT codes used were 47562, 47563, 47600, 47605, 47480, 47564, 47490, and 47570, which represent laparoscopic and open cholecystectomies (with or without an intraoperative cholangiogram), percutaneous or open cholecystostomies (with or without exploration, drainage, or removal of calculus), and cholecystenterostomies, respectively. The ICD-10 included were K81.9, K81.2, K81.1, K81.0, K80.80, K80.60, K80.50, K80.44, K80.20, K80.18, K80.12, K80.10, and K80.00 along with the corresponding ICD-9 (that matched the aforementioned ICD-10) codes. These ICD codes represent combinations of acute and chronic, obstructive and non-obstructive cholecystitis, cholelithiasis, cholangitis, and choledocholithiasis. All charges (which include any admission, clinic visit, urgent care visit, related procedures) were captured for each patient after April 2013 (*i.e.*, we were unable to obtain charge data for 23.5% of the cohort).

Patients were diagnosed with acute cholecystitis and stratified by severity using the latest TG18 criteria<sup>[10]</sup>. The diagnosis is based on local signs of inflammation (*e.g.*, Murphy's sign or right upper quadrant mass, pain, or tenderness), systemic signs of inflammation (*e.g.*, fever or elevated WBC), and characteristic imaging findings. TG18 categorizes acute cholecystitis by Grades 1, 2, and 3 representing mild, moderate, and severe, respectively.

### Statistical analysis

We conducted our evaluation by means of descriptive statistics using univariate and bivariate analyses of continuous and categorical variables. Bivariate comparison between the groups was analyzed using the Student's *t*-tests, one-way ANOVA, Chi-squared tests, and Fisher's exact tests, where appropriate. We constructed a multivariate model on the covariates potentially predictive of complications with a *P* value on bivariate comparison of  $\leq 0.2$ . A *P* value of  $< 0.05$  was considered statistically significant. Patient data was exported into a comma-separated value format to be imported in to R for analysis; all analyses were executed in the R statistical package (R, Developmental, Core, Team. R: A Language and Environment for Statistical Computing 2015; available at <http://www.R-project.org>).

## RESULTS

Five hundred fifty-four patients met criteria and were included in the study. Four hundred eighty-six (87.7%) underwent an SA cholecystectomy while 68 patients (12.3%) received delayed operative management. **Table 1** shows the demographics and patient characteristics of the two groups. Age, gender, race, ethnicity, body mass index, preexisting comorbidities, alcohol use, smoking status, and rates of planned or convert-to-open cholecystectomy were not significantly different between the SA and delayed cholecystectomy groups.

The cohort was stratified by the severity of acute cholecystitis based on the Tokyo grading system (**Table 2**). There were no differences in severity of disease between the SA and the delayed cholecystectomy groups.

Complication rates were significantly increased for SA compared to Delayed cholecystectomy for a diagnosis of acute cholecystitis (18.5% *vs* 4.4%, respectively,  $P = 0.004$ ; **Table 3**). Although complications in mild (Grade 1) and severe (Grade 3) acute cholecystitis were not significant, patients with moderate disease (Grade 2) experienced significantly more complications during SA compared to the delayed group (16.1% *vs* 0.0%,  $P < 0.001$ ). When we compared the complication rate of patients undergoing a delayed cholecystectomy, we found that the postoperative complication rate was similar to the rate for elective cholecystectomy for chronic cholecystitis (4.4% *vs* 6.2%,  $P = 0.557$ ). As expected, the overall complication rate of acute cholecystitis (combining both SA and delayed) was significantly greater than that of an elective cholecystectomy (16.8% *vs* 6.2%,  $P < 0.001$ ).

**Table 3** also shows the types of complications. A significantly increased rate of subtotal cholecystectomy was performed for the SA patients compared to delayed (7.6% *vs* 0.0%,  $P = 0.016$ ). Since a subtotal cholecystectomy was not the intended operation, we considered the performance of a subtotal cholecystectomy as a complication. Subtotal cholecystectomy, which is essentially a drainage procedure, was only performed in the SA group, suggesting that subtotal cholecystectomy is a surrogate for operative difficulty. A total of 37 patients underwent subtotal cholecystectomies; eight patients were Grade 1, 27 were Grade 2, and two patients were Grade 3. If we excluded subtotal cholecystectomies as a "true" complication, the complication rate was still statistically significant: 13.4% *vs* 4.4% (SA *vs* delayed, respectively,  $P = 0.03$ ). Mortality rates among all patients were 0.6% *vs* 0.0% for SA and delayed, respectively. All three patients who died had cholecystectomy within 24 h of admission from the emergency department. The first death occurred in a 90-year-old with Grade 3 disease. The second patient had Grade 1 disease and a history of congestive heart failure. The third had Grade 2 disease and unrecognized cardiac ischemia postoperatively. Notably, there were no common bile duct injuries in either group (**Table 3**). All other complication types identified were similar between the groups, including bile leak, retained stone, cholangitis, biliary stenosis, infection, hernia, and complications involving the cardiovascular, respiratory, or gastrointestinal systems.

The time between initial admission and operative intervention for the delayed cholecystectomy group was  $48.3 \pm 76.3$  d (**Table 4**). Postoperative readmission rates were similar between the groups (22.2% *vs* 21.2%,  $P = 0.854$ ). In comparison to the delayed cases, the SA group had significantly longer total lengths of hospital stay ( $3.8 \pm 5.5$  *vs*  $1.6 \pm 2.0$ ,  $P < 0.001$ ).

SA cholecystectomy hospital charges were higher compared to Delayed intervention for acute cholecystitis ( $\$44500 \pm \$59000$  *vs*  $\$35300 \pm \$16700$ ,  $P = 0.019$ , **Table 4**). Hospital charges for elective cholecystectomies performed on chronic conditions were the lowest at  $\$23000 \pm \$10300$  ( $P < 0.001$ , 1-way ANOVA comparing all three).

There were no differences in complication rates based on insurance status for SA

**Table 1** Demographics and patient characteristics

Variable	Same admission cholecystectomy	Delayed cholecystectomy	P value
	(n = 486), n (%) or mean $\pm$ SD	(n = 68), n (%) or mean $\pm$ SD	
Age at surgery	44.7 $\pm$ 16.0	45.2 $\pm$ 15.7	0.812
Female	280 (64.8)	49 (74.2)	0.132
Race/ethnicity			0.447
White	183 (42.5)	35 (53.0)	
Black	57 (13.2)	7 (10.6)	
Hispanic	185 (42.9)	23 (34.9)	
Other	6 (1.4)	1 (1.5)	
Body mass index	32.2 $\pm$ 15.6	32.1 $\pm$ 6.4	0.93
Comorbidities			
Coronary artery disease	13 (3.0)	2 (3.0)	1
Myocardial infarction	1 (0.2)	0 (0.0)	1
Diabetes	35 (8.1)	4 (6.1)	0.565
Hypertension	80 (18.5)	18 (27.3)	0.096
Chronic obstructive pulmonary disease	3 (0.7)	1 (1.5)	0.435
Alcohol use	134 (42.8)	27 (46.6)	0.598
Smoking status	100 (26.1)	20 (31.3)	0.39
Open cholecystectomy	8 (1.7)	3 (4.4)	0.142
Convert-to-open cholecystectomy	7 (1.4)	2 (2.9)	0.305

(17.0% insured *vs* 14.1% uninsured,  $P = 0.127$ ) or Delayed cholecystectomy (0.7% insured *vs* 0% uninsured,  $P = 1$ ). As expected, the vast majority of delayed cholecystectomies (97.1%) were insured compared to a lower number for SA patients (71.2%;  $P < 0.001$ ).

A logistic regression analysis was performed to determine if there are any independent variables that can predict a higher risk of complications. We stratified disease severity by TG13/18 and found that complication rates were only significantly different with the Grade 2 subset (Table 5). The odds of any complication occurring was 2.1x greater in patients with Grade 2 disease compared to those with Grade 1 (95%CI: 1.2-3.7,  $P = 0.007$ , Table 5). The odds of any complication occurring in Grade 2 or 3 was 2.3x greater in males compared to female patients (95%CI: 1.0-5.1,  $P = 0.047$ ).

Risk analysis (Table 6) of developing a complication in the SA *vs* delayed groups demonstrated a relative risk of 4.2x (95%CI: 1.4-12.9) and attributable risk of 14.1% (95%CI: 4.6-23.6). Number needed to harm (NNH) analysis indicated that, among eight patients (95%CI: 5.0-12.3) with acute cholecystitis undergoing SA cholecystectomy, one patient will suffer a complication. Notably, the attributable and NNH for Grades 2 or 3 patients were 28.3% (95%CI: 12.3-44.4) and 4 (95%CI: 2.9-4.6), respectively.

## DISCUSSION

Historically, the timing of performing cholecystectomy for a diagnosis of acute cholecystitis has been very controversial. A Cochrane systematic review with meta-analysis of six randomized clinical trials (1998-2003) compared early *vs* delayed laparoscopic cholecystectomy for patients with acute cholecystitis and concluded no significant difference between the groups with regards to immediate postoperative mortality, bile duct injuries, other serious adverse events, or rates of conversion to open cholecystectomy<sup>[14]</sup>. The only significant clinical outcome in favor of early cholecystectomy was a shorter hospital length of stay by four days. Multiple publications support early (SA) over delayed (interval) cholecystectomy based on equivalent morbidity and mortality, and an overall better economic profile<sup>[1-8,15-17]</sup>. However, the data from our institution over the past 8.5 years show that for the subset of Grade 2 patients, there is greater risk of morbidity and mortality with SA cholecystectomy. When a complication does occur, we found a concomitant longer length of hospital stay with an associated increase in hospital costs. When Grade 2 patients undergo delayed cholecystectomy, the rate of complications decreases to that

**Table 2 Stratification by disease severity using 2013/18 Tokyo guidelines, *n* (%)**

	Same admission cholecystectomy, <i>n</i> = 486	Delayed cholecystectomy, <i>n</i> = 68	<i>P</i> value
Acute cholecystitis severity			
Tokyo Grade 1	299 (61.5)	36 (52.9)	0.271
Tokyo Grade 2	174 (35.8)	31 (45.6)	
Tokyo Grade 3	13 (2.7)	1 (1.5)	
Tokyo criteria	mean $\pm$ SD	mean $\pm$ SD	
White blood cell count	11.8 $\pm$ 4.5	11.4 $\pm$ 3.8	0.605
Duration of complaints	1.9 $\pm$ 7.3	1.4 $\pm$ 3.2	0.578
Palpable tender mass in RUQ	8 (1.6)	1 (1.5)	1
Marked local inflammation	66 (13.6)	13 (19.1)	0.221
Creatinine	0.9 $\pm$ 0.8	0.7 $\pm$ 0.2	0.104
International normalized ratio	1.1 $\pm$ 0.2	1.4 $\pm$ 0.7	0.534
Platelet count	271.9 $\pm$ 73.8	282.6 $\pm$ 92.0	0.633
Cardiovascular dysfunction	2 (0.41)	0 (0.0)	1
Neurological dysfunction	0 (0.0)	0 (0.0)	1
Respiratory dysfunction	0 (0.0)	0 (0.0)	1
Other vitals and laboratory values			
Temperature ( $^{\circ}$ C)	36.7 $\pm$ 0.4	36.6 $\pm$ 0.4	0.068
Total bilirubin	1.0 $\pm$ 1.4	1.1 $\pm$ 1.7	0.852
Direct bilirubin	0.1 $\pm$ 0.6	0.5 $\pm$ 1.5	0.474
Aspartate aminotransferase	89.5 $\pm$ 181.6	64 $\pm$ 100.6	0.32
Alanine aminotransferase	94.9 $\pm$ 181.8	70.0 $\pm$ 96.4	0.32
Alkaline phosphatase	102.3 $\pm$ 48.6	111.4 $\pm$ 69.6	0.591
Amylase	87.6 $\pm$ 235.5	54 $\pm$ 5.3	0.112
Lipase	207.6 $\pm$ 704.7	477.2 $\pm$ 1474.5	0.478

RUQ: Right upper quadrant.

of elective cholecystectomies for subacute diagnoses (*e.g.*, biliary colic, gallbladders polyps). Due to the small sample size in Grade 3 patients, the logistic regression analysis (Table 5) did not identify Grade 3 patients at risk for greater complications and death when comparing operative timing. Although we cannot make definitive statements about Grade 3 patients in our study, multiple investigators with larger sample sizes do validate the highest morbidity and mortality for the highest grade of disease severity<sup>[12,18,19]</sup>.

Hernandez *et al*<sup>[18]</sup> validated TG13 grading among a cohort of 443 patients at the Mayo Clinic in Rochester, MN. The odds of postoperative complications For TG grade 2 and 3 were 1.8 times and 4.9 times the risk compared to grade 1 patients. Their finding for Grade 2 patients is similar to our study where Grade 2 patients had an odds ratio of 2.1 for complications compared to Grade 1 patients. Cheng *et al*<sup>[20]</sup> compared complication rates between 103 patients with acute cholecystitis of either Tokyo Grades 1, 2, or 3. Similar to our work, they noticed an increased surgical complication rate in Grade 2 (25.0%) compared to Grades 1 (5.6%) and 3 (0.0%). The low complication rate for Grade 3 patients in both Chen and our data are due to low sample size. Also in agreement with our findings, the literature has demonstrated an excess risk of complications in males undergoing cholecystectomy for acute cholecystitis<sup>[21-23]</sup> although the reason for the association between gender and complication risk with cholecystectomy remains unclear.

In our study, among the SA cholecystectomy patients, the mean time from admission to operation was 2.2  $\pm$  14.2 d, with an overall mortality rate of 0.6%. In a recent population-based study the 30-d operative mortality for all cholecystectomies (elective and emergent) performed for gallstone disease was 0.15% (72 deaths out of *n* = 47912 patients)<sup>[24]</sup>. They report that the risk of death for “acute” surgery compared with “planned” surgery was 10-fold higher (95%CI: 2.41-41.95). Patients with perioperative complications were 3.3-fold higher (95%CI: 1.74-6.15) for risk of death. Furthermore, patients between ages 50-70 and > 70 had a 2.12 (95%CI: 0.67-6.74) and a 7.04 (95%CI: 2.23-22.26) fold increase risk of death compared to patients < age 50, respectively. Among the three patients in our study who died postoperatively, two



**Table 3** Complication rates and types, *n* (%)

Variable	Same admission cholecystectomy ( <i>n</i> = 486)	Delayed cholecystectomy ( <i>n</i> = 68)	<i>P</i> value
Total patients with at least 1 complication	90 (18.5)	3 (4.4)	0.004 <sup>a</sup>
Tokyo Grade 1	37 (12.4)	3 (8.3)	0.480
Tokyo Grade 2	46 (26.4)	0 (0.0)	< 0.001 <sup>a</sup>
Tokyo Grade 3	7 (53.8)	0 (0.0)	1
Subtotal cholecystectomy	37 (7.6)	0 (0.0)	0.016 <sup>a</sup>
Death	3 (0.6)	0 (0.0)	1
Hepatobiliary			
Common bile duct injury	0 (0.0)	0 (0.0)	–
Bile leak	9 (1.9)	0 (0.0)	0.610
Retained stone	12 (2.5)	0 (0.0)	0.377
Cholangitis	2 (0.4)	0 (0.0)	1
Biliary stenosis	3 (0.6)	0 (0.0)	1
Infection			
Wound infection	10 (2.1)	0 (0.0)	0.620
Abscess	2 (0.4)	0 (0.0)	1
Sepsis	1 (0.2)	1 (1.5)	0.231
<i>Clostridium difficile</i> colitis	3 (0.6)	0 (0.0)	1
Intravenous catheter infection	1 (0.2)	0 (0.0)	1
Cardiovascular			
Hemorrhage	4 (0.8)	1 (1.5)	0.482
Hemorrhagic shock	1 (0.2)	0 (0.0)	1
Cardiogenic shock	1 (0.2)	0 (0.0)	1
Congestive heart failure exacerbation	3 (0.6)	0 (0.0)	1
Chest pain	1 (0.2)	0 (0.0)	1
Respiratory			
Pneumonia	4 (0.8)	1 (1.5)	0.482
Respiratory failure	2 (0.4)	0 (0.0)	1
Pulmonary embolism	1 (0.2)	0 (0.0)	1
Pulmonary edema	1 (0.2)	0 (0.0)	1
Pleural effusion	1 (0.2)	0 (0.0)	1
Gastrointestinal			
Pancreatitis	6 (1.2)	0 (0.0)	1
Gastroenteritis	1 (0.2)	0 (0.0)	1
Small bowel injury	1 (0.2)	0 (0.0)	1
Ileus	2 (0.4)	0 (0.0)	1
Hernia			
Ventral hernia	1 (0.2)	0 (0.0)	1
Incisional hernia	2 (0.4)	1 (1.5)	0.325
Other			
Seizure	1 (0.2)	0 (0.0)	1
Wound dehiscence	2 (0.4)	0 (0.0)	1
Reactive hydrocele	1 (0.2)	0 (0.0)	1

Level of significance was

<sup>a</sup>*P* < 0.05.

were in their 5th decade of life and one patient was in the 9th decade of life.

In a study by Zafar *et al*<sup>[25]</sup> using Nationwide Inpatient Sample data from 2005-2009, a multivariate analysis demonstrated that the odds of complications and death increased when cholecystectomy was performed between days 2-5 [mortality OR 1.26 (95%CI: 1.00-1.58)] and 6-10 d [mortality OR = 1.93 (1.38-2.69)] after admission, compared to days 0-1 (OR = 1). Based on this large dataset of with *n* > 95500 patients, the authors determined that the optimal time to perform cholecystectomy was less than 48 h, and that if the timing exceeds 48 h, the delay would result in higher



**Table 4** Hospital length of stay, postoperative readmissions, and charges

Variable	Same admission cholecystectomy, (n = 486), n (%) or mean $\pm$ SD	Delayed cholecystectomy, (n = 68), n (%) or mean $\pm$ SD	P value
Time to surgical admission (d)		48.3 $\pm$ 76.3	
Surgical admission length of stay (d)	3.4 $\pm$ 5.3	0.4 $\pm$ 1.0	< 0.001 <sup>a</sup>
Preoperative (d)	2.2 $\pm$ 14.2	0.0 $\pm$ 0.2	< 0.001 <sup>a</sup>
Postoperative (d)	1.2 $\pm$ 14.8	0.4 $\pm$ 0.9	0.204
Readmissions after cholecystectomy			
Patients with at least 1 readmission	96 (22.2)	14 (21.2)	0.854
Mean number of readmissions Among those readmitted	1.8 $\pm$ 1.5	1.4 $\pm$ 0.6	0.074
Total length of stay <sup>1</sup> (d)	3.8 $\pm$ 5.5	1.6 $\pm$ 2.0	< 0.001 <sup>a</sup>
Total hospital charges ( $\times$ \$1000) <sup>2</sup>	44.5 $\pm$ 59.0	35.3 $\pm$ 16.7	0.019 <sup>a</sup>

<sup>1</sup>Total length of stay includes index admission, elective operative admission for delayed cases, and all postoperative complication-related readmissions.

<sup>2</sup>Hospital charges for April 2013-August 2018 only. Level of significance was

<sup>a</sup>P < 0.05.

morbidity and mortality. Gutt *et al*<sup>[16]</sup> found that cholecystectomy within 24 h (mean 0.6 d) of admission had the lowest morbidity compared to delayed cholecystectomy 7–45 d (mean 25 d) after presentation; mortality was equivalent with one death in each group (0.33%). However, Brooks *et al*<sup>[26]</sup> did not find an association between the timing of SA operation (0 d, 1 d, 2 d, 3 d and  $\geq$  4 d from admission) with 30-d morbidity and mortality after risk adjustment when analyzing data from the American College of Surgeons National Surgical Quality Improvement Program database; this result may be due to a smaller sample size compared with Zafar's dataset. Although our cohort of patients generally underwent cholecystectomy close to the 48-h time-frame, it is noteworthy that all three deaths were operated on within 24 hours of admission. The mortality rates in Zafar's study were 0.2%, 0.6% and 1.7% for 0–1 d, 2–5 d and 6–10 d, respectively. Interestingly, while Zafar *et al*<sup>[25]</sup> advocate early cholecystectomy within 48 hours of admission, they note that the mortality rate for day 0 was higher at 0.42%; they hypothesized that some patients may be under-resuscitated at day 0 and therefore at higher risk for death.

In a large retrospective study from Japan and Taiwan, the 30-d mortality rate for among 2947 patients undergoing primary cholecystectomy for TG grades 1, 2, and 3 of acute cholecystitis were 0.3%, 0.4%, and 4.1%, respectively<sup>[19]</sup>. Joseph *et al*<sup>[27]</sup> presented contemporary data (n = 857) from a single institution and noted no mortality in Tokyo Grade 1 patients, 8 (0.5%) deaths in Grade 2 patients and 6 (1.8%) deaths in Grade 3 patients. Their patient population is a heterogeneous mix, with only 51% who had an elevated WBC and 45% with no ultrasonographic signs of acute cholecystitis. They included a significant number of patients categorized as having “acute on chronic” disease, who do not meet the TG clinical criteria for acute cholecystitis. Although their thesis was that the Tokyo criteria were not sensitive for acute cholecystitis, the inclusion a significant number of patients with normal WBC implies that they had a larger denominator in their reported mortality rate. The mortality rate would be higher with a smaller denominator (if only patients who meet the TG criteria for acute cholecystitis were included). The increase in death rate among the SA group in our study was not statistically significant when compared to the delayed group due to our small sample size; however, postoperative death for a benign condition concerns us sufficiently to revisit the optimal timing of surgical intervention for acute cholecystitis.

To manage “difficult” gallbladders, the WSES recommends subtotal cholecystectomy especially in the acute setting or a delayed cholecystectomy 45 d from symptom onset. We defined a delayed cholecystectomy for acute cholecystitis as a procedure occurring after discharge electively after stabilization of acute disease. However, the literature does not have an agreed upon definition of a “delayed” cholecystectomy and ranges from 1 d to 6 wk after symptom onset, diagnosis, or index presentation. On average, patients in this study received a delayed, elective cholecystectomy 48.3 d from index presentation of acute disease. Our work supports the 2016 WSES recommendations for subtotal cholecystectomies and delayed time frame<sup>[1]</sup>.

A limitation of this study is the retrospective nature of our data; there is selection

**Table 5** Logistic regression analysis

Variable	OR	95%CI	P value
Occurrence of any complication - all patients			
Same admission cholecystectomy	0.7	0.1-7.7	0.759
Grade 2	2.1	1.2-3.7	0.007 <sup>a</sup>
Grade 3	2.0	0.4-10.2	0.404
Male	1.4	0.8-2.5	0.262
Diabetes	1.2	0.5-3.1	0.722
Hypertension	1.1	0.5-2.4	0.773
Open cholecystectomy	1.1	0.2-5.4	0.863
Insurance status	0.6	0.3-1.2	0.180
Age	1.0	1.0-1.0	0.919
Creatinine	0.9	0.7-1.4	0.780
Occurrence of any complication - Grade 1 patients			
Same admission cholecystectomy	0.2	0.0-1.3	0.094
Diabetes	2.5	0.7-9.0	0.157
Insurance status	0.3	0.1-1.0	0.052
Body mass index	1.1	1.0-1.1	0.048 <sup>a</sup>
Occurrence of any complication - Grade 2 patients			
Male	1.9	0.7-5.4	0.238
Smoking status	2.1	0.8-5.7	0.156
Open cholecystectomy	2.1	0.1-31.8	0.598
Age	1.0	1.0-1.0	0.975
Body mass index	1.0	1.0-1.1	0.277
Postoperative length of stay	1.4	1.0-2.1	0.056
Aspartate aminotransferase	1.0	1.0-1.0	0.848
Alanine aminotransferase	1.0	1.0-1.0	0.481
Lipase	1.0	1.0-1.0	0.482
Creatinine	2.8	0.3-30.7	0.402
Occurrence of any complication - Grade 2 or 3 patients			
Male	2.3	1.0-5.1	0.047 <sup>a</sup>
Open cholecystectomy	1.3	0.2-9.5	0.767
Age	1.0	1.0-1.0	0.326
Body mass index	1.0	1.0-1.0	0.565
Preoperative length of stay	0.8	0.6-1.0	0.061
Creatinine	1.0	0.7-1.4	0.919

Level of significance was

<sup>a</sup>P < 0.05. OR: Odds ratio; CI: Confidence interval.

bias especially for Grade 3 patients, where the small sample size precluded definitive conclusions. Since we did not include preoperative percutaneous cholecystostomy tubes, we likely excluded a significant number of Grade 3 patients from this study. Additionally, hospital charge data for the SA and delayed represent 78.4% and 69.1% of the total patients, respectively. Financial comparisons were made on the majority of patients, but would be more reliable if we could obtain all of the data. Finally, this study represents the experience at one academic, teaching institution with 26 faculty members performing the cholecystectomies. The faculty members are board-certified general surgeons with competency for laparoscopic and open cholecystectomy; only a few self-identify as having specific hepatobiliary or advanced laparoscopic expertise. We have a general surgery training program and all surgeons and surgical residents on these cases have earned Fundamentals of Laparoscopic Surgery-certification. The applicability of this study may not be generalizable in all practice settings.

Our work supports the 2018 TG for the management of Grades 1 and 2 acute cholecystitis<sup>[10]</sup>. Specifically, if a patient has significant co-morbidities and/or is a high-risk surgical candidate, conservative management with antibiotics, fluid resuscitation, and pain control should be followed by elective cholecystectomy. If the

**Table 6** Relative risk, attributable risk, and number needed to harm analysis

Variable	Risk	95%CI
All, SA <i>vs</i> delayed		
Relative risk	4.2x	1.4 to 12.9 <sup>a</sup>
Attributable risk	14.1%	4.6 to 23.6 <sup>a</sup>
Number needed to harm	8	5.0 to 12.3 <sup>a</sup>
Grade 1, SA <i>vs</i> delayed		
Relative risk	1.7x	0.5 to 5.1 <sup>a</sup>
Attributable risk	4.9%	-5.7 to 15.5
Number needed to harm	21	-7.2 to 24.4
Grade 2, SA <i>vs</i> delayed		
Relative risk <sup>1</sup>	-	-
Attributable risk	26.4%	13.2 to 39.7 <sup>a</sup>
Number needed to harm	4	3.0 to 5.0 <sup>a</sup>
Grade 2 or 3 <sup>2</sup> , SA <i>vs</i> delayed		
Relative risk <sup>1</sup>	-	-
Attributable risk	28.3%	12.3 to 44.4 <sup>a</sup>
Number needed to harm	4	2.9 to 4.6 <sup>a</sup>

<sup>1</sup>Relative risk analysis could not be performed in Grades 2 or 3 due to zero complications in the delayed group.

<sup>2</sup>Due to a small sample size of the rarer Grade 3 disease, combined analysis of Grades 2 and 3 was performed to represent all moderate and severe cases.

<sup>a</sup>Statistically significant. CI: Confidence Interval; SA: Same admission.

patient does not improve clinically with conservative management, percutaneous cholecystostomy can be performed to drain the septic source. In this study, we have compared the clinical and economic outcomes of SA cholecystectomy with delayed cholecystectomy for acute cholecystitis. Early cholecystectomy for Tokyo Grades 2 and 3 patients should be considered with care. The risks of complications are higher in these patient groups, leading to greater cost. Future studies are needed to confirm our findings and also should focus on strategies to avoid mortality in these high-risk groups.

In conclusion, patients presenting with acute cholecystitis Tokyo Grade 2 developed more complications and incur increased charges when undergoing SA cholecystectomy compared to a delayed approach. This data supports a selective approach to surgery for patients with acute cholecystitis; Tokyo Grade 2 patients have a lower complication rate when cholecystectomy is delayed.

## ARTICLE HIGHLIGHTS

### Research background

The timeframe of when to perform cholecystectomy for acute cholecystitis has been controversial for years. Most recently, clinical practice has favored operative intervention during the same admission (SA) (early cholecystectomy). We present a comparison of complications between SA *vs* interval (delayed) cholecystectomy.

### Research motivation

Recent enthusiasm for SA cholecystectomy is based on projected economic advantage. We hypothesized that the economic advantage may be lost if complication rates are higher than expected.

### Research objectives

We compared the complication rates and hospital charges between SA *vs* delayed cholecystectomy patients. Patients were stratified by Tokyo Grade.

### Research methods

We performed a retrospective chart review of all patients at a single institution who presented for cholecystectomy due to acute cholecystitis between February 2010 through August 2018. Hospital charges were also obtained when available. Descriptive statistics were used to compare the groups; a multivariate model on the covariates predicting complications was also performed.

### Research results

SA cholecystectomy patients had an overall complication rate of 18.5% compared to Delayed cholecystectomy patients with a complication rate of 4.4% ( $P = 0.004$ ). For the Tokyo Grade 2 patients (moderate disease), SA and delayed cholecystectomy complication rates were 16% *vs* 0%, respectively ( $P < 0.001$ ). SA cholecystectomy hospital charges were higher compared to Delayed cholecystectomy ( $P = 0.019$ ) due to an increase in cost from the management of complications. There were no significant differences in clinical outcomes for Tokyo Grade 1 patients (mild disease). We did not have sufficient numbers of patients with Tokyo Grade 3 (severe disease) for meaningful comparisons.

### Research conclusions

Our study demonstrates that SA cholecystectomy patients have higher complication rates with associated higher costs. The data supports a selective approach to operative intervention for acute cholecystitis; Tokyo Grade 2 patients have a lower complication rate when cholecystectomy is Delayed. Risk factors for complications include Tokyo Grade 2 severity of disease. In a risk analysis, among eight patients with acute cholecystitis undergoing SA cholecystectomy, one patient will suffer a complication.

### Research perspectives

This study suggests that SA cholecystectomy does not always afford an economic advantage, especially if there are complications. Future studies are needed to confirm our findings since this study is limited because the data was collected retrospectively from a single institution.

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## Observational Study

## Hepatitis C virus eradication with directly acting antivirals improves health-related quality of life and psychological symptoms

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**Institutional review board**

**statement:** This study protocol was reviewed and approved by the the Ethical and the Research Committees of the "Sapienza" University of Rome.

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## Abstract

## BACKGROUND

Alterations in health-related quality of life (HRQoL) and neuropsychological disorders were described in the hepatitis C virus (HCV) patients. Although several studies investigated the modifications of HRQoL after HCV eradication, no data exists on the modifications of neuropsychological symptoms.

## AIM

To investigate the effect of directly acting antivirals (DAAs) treatment on HRQoL and neuropsychological symptoms.

## METHODS

Thirty nine patients with HCV infection underwent a neuropsychological assessment, including Zung-Self Depression-Rating-Scale, Spielberg State-Trait Anxiety Inventory Y1-Y2 and the Toronto-Alexithymia Scale-20 items before and after DAAs treatment. HRQoL was detected by Short-Form-36 (SF-36).

## RESULTS

All HRQoL domains, but role limitation physical and bodily pain, significantly improved after treatment. Interestingly, after DAAs treatment, all domains of HRQoL returned similar to those of controls. Each neuropsychological test significantly improved after HCV eradication. A significant correlation was observed among each psychological test and the summary components of SF-36. At multiple linear regression analysis including each psychological test as possible covariates, Zung-Self Depression Rating Scale (Zung-SDS) score was independently and significantly related to summary components of the SF-36 in the basal state and the difference between Zung-SDS score before and after treatment was the only variable significantly and independently related to the modification of HRQoL induced by the treatment.



revised according to the STROBE Statement-checklist of items.

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## CONCLUSION

Neuropsychological symptoms strongly influenced HRQoL in HCV patients and there was a significant improvement of neuropsychological tests and HRQoL after DAAs treatment.

**Key words:** Hepatitis C virus; Health related quality of life; Depression; State and trait anxiety; Alexithymia; Directly acting antivirals

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**Core tip:** In patients with the hepatitis C virus (HCV) infection, alterations in health-related quality of life (HRQoL) and neuropsychological disturbances were described also in the absence of liver cirrhosis. During the last years, HCV therapy has evolved from interferon-based to directly acting antiviral (DAA)-based therapy, with excellent tolerability and efficacy. Until now, few data exist on the modifications of neuropsychological symptoms before and after DAAs treatment and on the relationship of these symptoms on HRQoL. With this study we demonstrated that HCV eradication with DDAs treatment significantly improves health-related quality of life and neuropsychological symptoms.

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## INTRODUCTION

The hepatitis C virus (HCV) infection is one of the most frequent causes of liver cirrhosis in the Western World with consequent significant social and health burden<sup>[1]</sup>. It is known that patients with chronic medical illness, such as liver cirrhosis, may be afflicted by symptoms of depression<sup>[2]</sup> anxiety<sup>[3]</sup> and alexithymia<sup>[4,5]</sup>, especially in the end stage of their liver disease, which is characterized by frequent medical complications, hospitalization, functional limitation and change of body image. In patients with HCV infection, alterations in health-related quality of life (HRQoL) and neuropsychological disturbances were described also in the absence of liver cirrhosis<sup>[6,7]</sup>. In fact, even in the absence of debilitating symptoms, HCV may adversely affects the HRQoL by negatively impacting on the physical and mental well-being of the patients<sup>[8]</sup>. Nonspecific symptoms such as asthenia, irritability, general malaise, muscle and joint aches and headaches may occur in HCV patients<sup>[9]</sup>. In most cases these symptoms do not require medical intervention but affect the sense of physical well-being and cause emotional problems. It has been suggested that the virus may impact on cognitive functions thereby evoking abnormal changes in sleep patterns and attention deficit regardless of the stage of the disease<sup>[10,11]</sup>. Finally, apprehension for themselves and for the patients' family may contribute to the development of anxiety and depressive symptoms.

Many authors have postulated a direct action of the HCV virus at the CNS level and viral replicates have been detected in the cerebral tissue but it is difficult to determine whether the neuropsychiatric symptoms are due to a direct neurotoxicity of the virus per se or to emotional stress related to functional deficit, social stigma and to apprehension for long-term prognosis<sup>[10,11]</sup>.

In the past, patients with a diagnosed mental health disease (MHD), defined as either a Diagnostic and Statistical Manual of Mental Disorders (DSM) (DSM IV) diagnosis of major depression, bipolar disorder, schizophrenia, generalized anxiety, and post-traumatic stress disorder or requiring anti-depressants, antipsychotics, mood stabilizers or psychotropic prescribed by a psychiatrist, were marginalized with respect to HCV therapy and MHD was one of the most frequently cited reason for exclusion from HCV interferon-based therapy<sup>[12]</sup>. During the last years, HCV therapy has evolved from interferon-based to directly acting antiviral (DAA)-based therapy, with excellent tolerability and efficacy<sup>[13]</sup>. Although several studies investigated the modifications of HRQoL after HCV eradication<sup>[14-18]</sup>, until now, to our knowledge, no

data exists on the modifications of neuropsychological symptoms (depression, anxiety and alexithymia symptoms) before and after directly acting antivirals (DAAs) treatment and on the relationship of these symptoms on HRQoL. Thus, aim of the present study was to investigate the effect of HCV infection on HRQoL and neuropsychological symptoms and to analyse the modifications of these parameters after DAAs treatment.

## MATERIALS AND METHODS

From January 2018 to January 2019, thirty-nine patients with HCV infection submitted to Ledipasvir/Sofosbuvir treatment, were consecutively enrolled at “Santa Maria Goretti” Hospital of Latina, Sapienza University of Rome. The diagnosis of HCV infection was based on history, clinical examination, biochemical, elastography and ultrasound findings. Liver fibrosis was quantified according to Metavir staging system<sup>[19]</sup>, based on Fibroscan® score. Exclusion criteria were: HIV co-infection; presence of a previously diagnosed mental health disease, defined as either a DSM IV diagnosis of major depression, bipolar disorder, schizophrenia, generalized anxiety, and post-traumatic stress disorder or requiring anti-depressants, antipsychotics, mood stabilizers or psychotropics prescribed by a psychiatrist; alcohol abuse in the last 3 mo; other causes of liver disease different from HCV infection.

An informed written consent to participate to the study was obtained by all the patients. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethical and the Research Committees of the “Sapienza” University of Rome.

### Psychological assessment

All neuro-psychological assessment was carried on by an expert (D.R.) who administered the questionnaires. Depressive symptoms were assessed by the Zung-Self Depression Rating Scale (Zung-SDS)<sup>[20]</sup>, which includes 20 items with negative and positive contents. Patients have to mark the answer that best suits their present state of mind. Each answer is given 1 to 4 points. A higher total score corresponds to the presence of depressive symptoms; the cut-off is 50 out of 80.

Anxiety symptoms were detected by the Spielberg State-Trait Anxiety Inventory (STAI)Y1-Y2<sup>[21]</sup>, a widely used anxiety rating scale. It consists of 40 items, each graded from 1 to 4. The scale differentiates anxiety into (1) anxiety caused by a specific condition (state anxiety); (2) anxiety as a more permanent patient disorder (trait anxiety). The main variables that the questionnaire measures are anxiety, apprehension, nervousness and tension. A high score is associated with higher anxiety level; the cut-off is 60 points out of 80.

Alexithymic symptoms were detected by 20-item Toronto alexithymia scale (TAS-20)<sup>[22]</sup>. This test comprises three scales: Difficulty identifying feelings (DIF: Seven items), difficulty describing feelings (DDF: Five items), and Externally-Oriented Thinking (EOT: Eight items). Items are rated using a 5-point Likert scale whereby 1 = strongly disagree and 5 = strongly agree. The total alexithymia score is the sum of responses to all 20 items, the cut-off is a score > 60.

### Evaluation of health-related quality of life

HRQoL was evaluated with Short Form-36<sup>[23]</sup>, a paper-pencil questionnaire that consists of 36 multiple-choice questions. It measures eight domains, four in the area of “physical health” (physical functioning, role limitation-physical, bodily pain, general health) and four in the area of “mental health” (role limitation-emotional, vitality, mental health and social functioning). Each domain earns between 0 and 100 points, with higher scores indicating a better HRQoL. Two comprehensive indices of HRQoL may also be computed: The physical component summary (PCS) and the mental component summary (MCS). The Short-Form-36 (SF-36) was evaluated according to the Apolone *et al*<sup>[24]</sup>’s instructions, using a free, downloadable software which allows each patient to be compared with one healthy Italian man or woman. With this software we obtain control values of healthy Italian population matched for age, gender, degree of education, job and marital status. All questionnaires were administered before DAAs treatment and after the evidence of SVR, defined as undetectable viral load 12 weeks after the end of the therapy.

### Statistical analysis

Data are presented as means ± SD. Comparisons among groups and before and after DAA treatment were performed by unpaired or paired Student-*t*-test or paired  $\chi^2$  for quantitative and qualitative data, respectively. For continuous variables that showed abnormal distribution Wilcoxon Signed-Rank Test was used, likewise McNemar Test

was used to compare nonparametric dependent groups. The relationship between the scores of each psychological test and the PCS or MCS of SF-36 was evaluated by Pearson's correlation coefficients.  $P < 0.05$  were considered statistically significant. Two multiple linear regression analyses were used to identify the variables related to the component summary of SF-36 at basal state and to its variations before and after treatment (*i.e.* the difference between the final and initial value of each test). Zung-SDS, TAS-20, STAI Y1 and STAI Y2 scores were included in the multivariate analyses. All the analyses will be performed using the SPSS software Version 16.0.

## RESULTS

The sample included 39 patients with HCV infections, 23 men and 16 women; among them 9 patients were classified as affected by well compensated cirrhosis (7 Child-Pugh class A and 2 Child-Pugh class B) on clinical and radiological findings and on the degree of the fibrosis according to Metavir score = F4. Comparing patients with and without cirrhosis there was no statistical difference in albumin levels ( $3.75 \pm 0.59$  vs  $4.10 \pm 0.38$  g/dL;  $P = 0.15$ ), bilirubin levels ( $1.04 \pm 0.36$  vs  $0.67 \pm 0.29$  mg/dL;  $P = 0.09$ ), INR ( $1.17 \pm 0.54$  vs  $0.98 \pm 0.16$ ). Given the similarity of the two groups and the small number of the patients, we decided to consider all patients as a single group.

The mean age of the patients was  $59.8 \pm 14.2$  years; twenty-five of these had genotype 1, five patients genotype 2, seven patients genotype 3 and two patients genotype 4. Only 14 patients were naive, the remaining twenty five patients were previously submitted to IFN-based regimens. The main clinical and demographic features of the patients are reported in Table 1. After DAAs treatment, all patients achieved sustained virological response and, as expected, there was a significant amelioration of serum transaminases: ALT (U/L)  $75.1 \pm 77.8$  vs  $26 \pm 7.1$ ,  $P < 0.001$ ; AST (U/L)  $101.9 \pm 86.5$  vs  $27.3 \pm 5.5$ ,  $P < 0.001$ .

The comparison between HRQoL domains of controls and patients before and after treatment with DAAs is shown in Figure 1. As widely described in literature, also in our sample the quality of life was significantly worse in HCV patients compared to controls values. The results of SF-36 before and after HCV eradication are shown in Figure 1 and Table 2. All HRQoL domains, but role limitation physical, bodily pain and social functioning, significantly improved after DAAs treatment. Interestingly, after DAAs treatment, all domains of HRQoL returned similar to those of healthy controls (Figure 1).

The results of Zung-SDS, STAI Y1-Y2 and TAS-20 before and after DAAs treatment are reported in Table 3. A significant improvement after DAAs treatment was observed for each test. According to the cut of value of each test, 8/39 (20%) had depressive symptoms (Zung  $\geq 50$ ), 2/39 (5%) had symptoms related to state anxiety (STAI-Y2  $\geq 60$ ), 4/39 (10%) symptoms related to trait anxiety (STAI-Y1  $\geq 60$ ), and 11/39 (28%) had alexithymia (TAS-20  $\geq 60$ ) at basal evaluation. The prevalence of neuropsychological disorders decreased after HCV eradication without reaching the statistical significance (Table 4).

A significant correlation between each neuropsychological test and the two components of HRQoL (PCS and MCS) at basal evaluation was found (Figure 2 and Table 5). Significant correlations were also observed between the variations of Zung-SDS, TAS-20, STAI-Y1 and Y2 and HRQoL components (*i.e.*, the difference between the final and initial value of each test) before and after DAAs treatment, as shown in Figure 3 and Table 6.

Multiple linear regression analysis was used to identify the possible covariates related to the physical and mental component of the SF-36. Scores tests evaluating depression, anxiety and alexithymia symptoms were included in the analysis as possible covariates. Zung score was independently and significantly related to both the mental and physical component of the SF-36 in the basal state and the difference between Zung score before and after treatment was the only variable significantly and independently related to the modification of HRQoL induced by the treatment (Table 7).

## DISCUSSION

It is estimated that approximately 71 million individuals are infected with HCV worldwide<sup>[1]</sup>. Diagnosis of HCV infection can significantly affect the patients' social life. In the decompensated state of the disease, due to the inability to hold down a job, many HCV patients experience financial difficulties along with a sensation of awkwardness, social denial and all of this may lead to a worsening of their

**Table 1 Clinical and demographic characteristics of the patients**

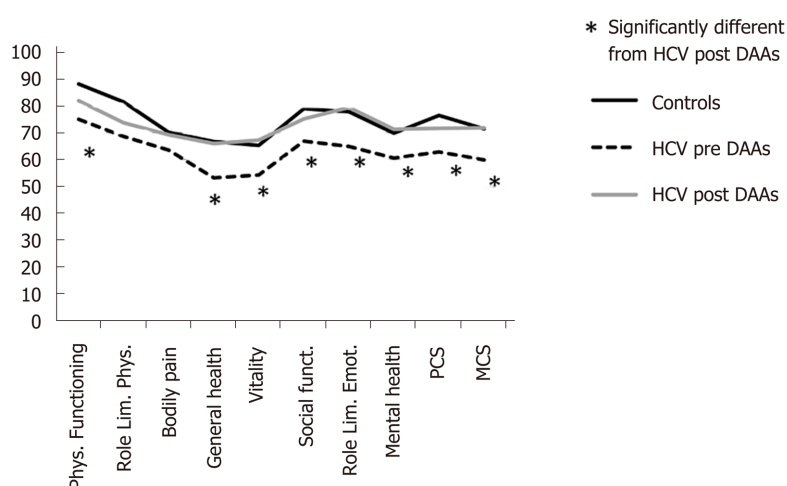
Characteristic	Data
Men/Women	23/16
Age (yr)	59.8 ± 14.2
Hepatitis C virus-RNA (UI/mL)	1.624 × 10 <sup>3</sup> ± 1.522 × 10 <sup>3</sup>
Hepatitis C virus genotype (n)	
1a/1b	4/21
2	5
3	7
4	2
Fibrosis according to Metavir (n)	
F0-F2	21
F3-F4	18

socioeconomic status and quality of life<sup>[25]</sup>.

As previously demonstrated in a cohort of cirrhotic patients<sup>[5]</sup>, among the factors that could influence HRQoL there are psychological symptoms such as anxiety, depression and alexithymia. Even in the absence of decompensated cirrhosis, patients with chronic HCV infection, can develop an altered emotional status as well as anxiety and depressive symptoms. Many unfounded reasons could be involved: The awareness of having a chronic disease potentially with a poor long-term prognosis, the shame for having been infected, the fear of infecting loved ones, the guilt of having an infection which still today may represent a social stigma as sexually transmitted or substances abuse diseases. Moreover, until a few years ago, the patients were also worried about the limited eradication capacity of interferon based regimens and by their innumerable side effects<sup>[25]</sup>. The present study confirms that the HRQoL is impaired in HCV population as already observed<sup>[8,12-16,25]</sup> even in patients with compensated disease. Fortunately, the emergence of DAAs, with high efficacy rates and tolerability profile, have completely revolutionized the treatment of chronic HCV treatment<sup>[13]</sup>.

Health-related quality of life is a key component in the evaluation of any therapeutic intervention. It may be more relevant than length of life, because patients are frequently more concerned about quality and disability than about longevity<sup>[26]</sup>. Several studies have already investigated the modifications of HRQoL after HCV eradication with the new IFN-free regimen<sup>[14]</sup>. In particular the trial conducted by Ng *et al*<sup>[17]</sup> demonstrated that HCV treatment with Elbasvir/Grazoprevir resulted in a significant improvement of patients reported outcomes compared to Sofosbuvir/Peg-IFN/Ribavirin regimen. Another Brazilian study<sup>[18]</sup> evaluated the modifications of HRQoL measured with SF36 and CLDQ in a cohort of 56 HCV patients treated with different DAAs regimens and they found a significant amelioration of both tests after HCV eradication. These results were confirmed in the present study. In fact, all domains of HRQoL, but role limitation physical and bodily pain, evaluated after the end of therapy significantly improved after DAAs treatment. Interestingly, differently from that observed in the study of Juanbeltz *et al*<sup>[18]</sup> in which HRQoL remains lower than that of general population despite viral clearance, in our population HRQoL after HCV eradication returned similar to that of healthy controls. These different results may be related to the greater number of cirrhotics enrolled in the Juanbeltz *et al*<sup>[18]</sup> study.

Especially in patients with HCV related compensated disease the improvement of HRQoL observed after HCV eradication could be likely due to an amelioration of the emotional and psychological well being. Therefore, the hypothesis of the present study was to test the role of emotional status evaluated by standardized tests able to detect symptoms of anxiety, depression and alexithymia on HRQoL in HCV patients. A second aim was to relate the modification of HRQoL to those of neuropsychological test after DAA treatment. At basal evaluation, significant correlations were found between all neuropsychological test and component summary of HRQoL. When the questionnaires used to evaluate anxiety, depression and alexithymia were included in a multivariate analysis, Zung score, a measure of depressive tract, was the only variable independently correlated to both physical and mental component of HRQoL, confirming the hypothesis that chronic HCV infection could induce the occurrence of depressive symptoms. Moreover and more interestingly, we found a significant improvement of neuropsychological tests after HCV eradication and a significant



**Figure 1 Comparison between Short-Form-36 domains of controls and patients before and after hepatitis C virus eradication.** HCV: Hepatitis C virus; DAAs: Directly acting antivirals; MCS: Mental component summary; PCS: Physical Component Summary.

correlation between the modification of these tests and of HRQoL, after therapy. As for the basal state the modification of the Zung score was the major determinant of the variation of the HRQoL. To our knowledge, no study investigated the modifications of depression, anxiety and alexithymia symptoms before and after DAAs treatment.

The study has some limitation: The sample size is limited and the majority of patients were affected by chronic hepatitis or very well compensated cirrhosis. Consequently the conclusion on the relationship between the modification of neuropsychological disorders and HRQoL cannot be extended to more advanced stage of HCV disease. Moreover, all patients were treated with a single regimen (Ledipasvir/Sofosbuvir). Thus, the observed effects cannot be formally extended to other treatments. On the other hand, the homogeneity of the study is higher than that obtainable using different drugs.

In conclusion, depression, state and trait anxiety and alexithymia symptoms are among the major determinants of the altered HRQoL, even in patients with chronic HCV infection, without liver cirrhosis. Therefore, the detection of these symptoms appears to be important when the patients' health status perception has to be considered.



**Table 2 Health related quality of life domains before and after directly acting antivirals treatment**

	Pre DAAs (n = 39)	Post DAAs (n = 39)	P value
Physical functioning	75 ± 27.3	81.9 ± 22.2	0.005
Role limitation physical	68.6 ± 40.4	73.7 ± 36.3	0.35
Bodily pain	63.5 ± 32.2	69.2 ± 27.1	0.19
General health	53.1 ± 21.2	65.9 ± 17.5	< 0.001
Vitality	54.2 ± 20.4	67.2 ± 18.1	< 0.001
Social functioning	66.8 ± 25.4	75.1 ± 20.6	0.03
Role limitation emotional	64.8 ± 39.7	79.4 ± 34.7	0.02
Mental health	60.5 ± 22.2	71.2 ± 18.9	0.002
Physical component summary	62.8 ± 22.7	71.6 ± 21.2	0.002
Mental component summary	59.8 ± 21.9	71.8 ± 19.1	< 0.001

Paired *t* test, mean ± SD. DAAs: Directly acting antivirals.

**Table 3 Neuropsychological tests before and after directly acting antivirals treatment**

	Pre DAAs (n = 39)	Post DAAs (n = 39)	P value
TAS-20	49.2 ± 14.2	42.5 ± 12.6	< 0.001
Zung-SDS	41.7 ± 9.3	37.1 ± 8.6	< 0.001
STAI-Y1	39.8 ± 11.9	32.8 ± 7.8	< 0.001
STAI-Y2	41.4 ± 12.2	35.7 ± 9.2	< 0.001

Paired *t* test, mean ± SD. DAAs: Directly acting antivirals; Zung-SDS: Zung-Self Depression-Rating-Scale; STAI-Y1: Spielberg State-Trait Anxiety Inventory Y1; STAI-Y2: Spielberg State-Trait Anxiety Inventory Y2; TAS-20: Toronto-Alexithymia Scale-20.

**Table 4 Prevalence of depression, state and trait anxiety and alexithymia before and after directly acting antivirals treatment, n (%)**

	Pre DAAs (n = 39)	Post DAAs (n = 39)	P value
Zung SDS ≥ 50 (no/yes)	8/31 (20)	4/35 (10)	Ns
STAI Y1 ≥ 60 (no/yes)	37/2 (5)	39/0 (0)	Ns
STAI Y2 ≥ 60 (no/yes)	35/4 (10)	39/0 (0)	Ns
TAS-20 ≥ 60 (no/yes)	28/11 (28)	34/5 (13)	Ns

Paired  $\chi^2$ . DAAs: Directly acting antivirals; Zung-SDS: Zung-Self Depression Rating Scale; STAI-Y1: Spielberg State-Trait Anxiety Inventory Y1; STAI-Y2: Spielberg State-Trait Anxiety Inventory Y2; TAS-20: Toronto-Alexithymia Scale-20; SF36: Short form 36.

**Table 5 Correlation matrix between neuropsychological tests and physical component summary and mental component summary of health-related quality of life at basal evaluation**

	PCS	MCS	TAS-20	Zung	STAI-Y1	STAI-Y2
PCS	1	0.886 <sup>b</sup>	-0.343 <sup>a</sup>	-0.735 <sup>b</sup>	-0.314 <sup>a</sup>	-0.471 <sup>b</sup>
MCS	0.886 <sup>b</sup>	1	-0.434 <sup>b</sup>	-0.775 <sup>b</sup>	-0.534 <sup>b</sup>	-0.622 <sup>b</sup>
TAS-20	-0.343 <sup>a</sup>	-0.434 <sup>b</sup>	1	0.349 <sup>a</sup>	0.415 <sup>b</sup>	0.726 <sup>b</sup>
Zung	-0.735 <sup>b</sup>	-0.775 <sup>b</sup>	0.349 <sup>a</sup>	1	0.586 <sup>b</sup>	0.634 <sup>b</sup>
STAI-Y1	-0.314 <sup>a</sup>	-0.534 <sup>b</sup>	0.415 <sup>b</sup>	0.586 <sup>b</sup>	1	0.686 <sup>b</sup>
STAI-Y2	-0.471 <sup>b</sup>	-0.622 <sup>b</sup>	0.726 <sup>b</sup>	0.634 <sup>b</sup>	0.686 <sup>b</sup>	1

<sup>a</sup>*P* < 0.05;

<sup>b</sup>*P* < 0.01. DAAs: Directly acting antivirals; Zung-SDS: Zung-Self Depression-Rating-Scale; STAI-Y1: Spielberg State-Trait Anxiety Inventory Y1; STAI-Y2: Spielberg State-Trait Anxiety Inventory Y2; TAS-20: Toronto-Alexithymia Scale-20; SF-36: Short Form 36; PCS: Physical component summary; MCS: Mental component summary.



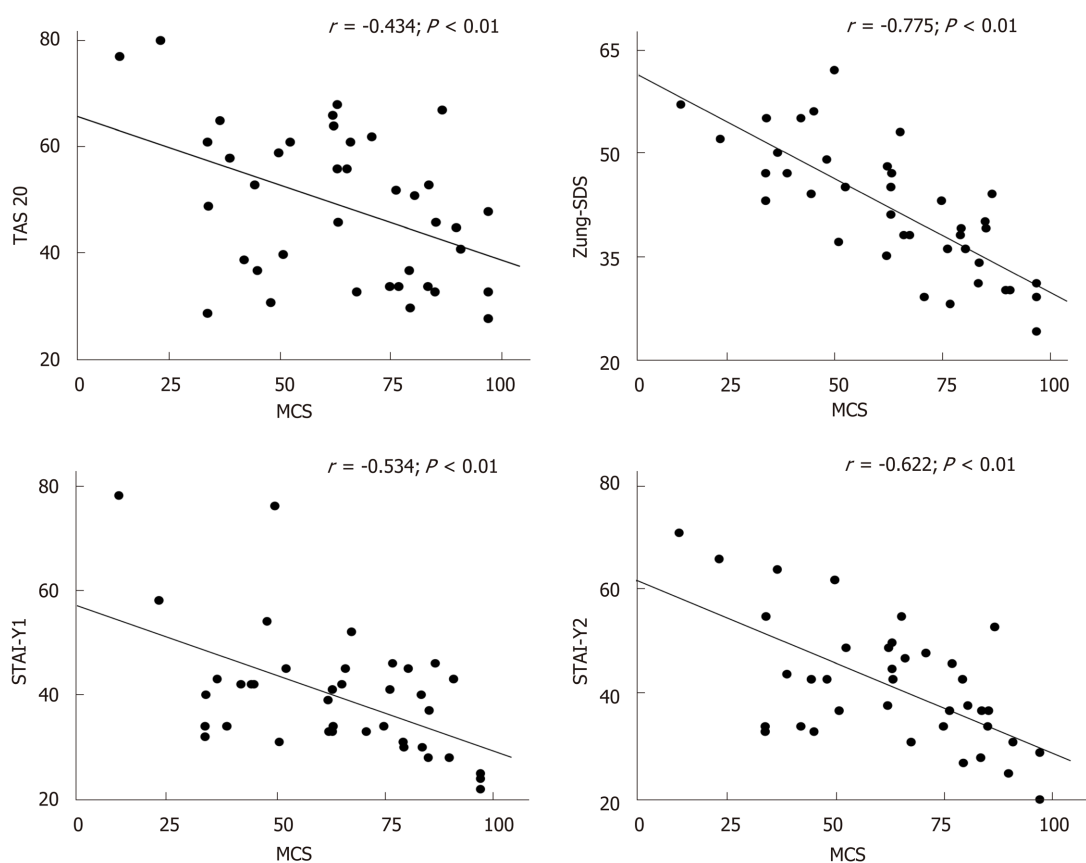
**Table 6 Correlation matrix between the variations of each neuropsychological test and physical component summary and mental component summary before and after directly acting antivirals treatment**

	$\Delta$ PCS	$\Delta$ MCS	$\Delta$ TAS-20	$\Delta$ Zung	$\Delta$ STAI-Y1	$\Delta$ STAI-Y2
$\Delta$ PCS	1	0.752 <sup>b</sup>	-0.329 <sup>a</sup>	-0.741 <sup>b</sup>	-0.218	-0.533 <sup>b</sup>
$\Delta$ MCS	0.752 <sup>b</sup>	1	-0.494 <sup>b</sup>	-0.775 <sup>b</sup>	-0.469 <sup>b</sup>	-0.654 <sup>b</sup>
$\Delta$ TAS-20	-0.329 <sup>a</sup>	-0.494 <sup>b</sup>	1	0.307	0.257	0.539 <sup>b</sup>
$\Delta$ Zung	-0.741 <sup>b</sup>	-0.775 <sup>b</sup>	0.306	1	0.395 <sup>a</sup>	0.603 <sup>b</sup>
$\Delta$ STAI-Y1	-0.218	-0.469 <sup>b</sup>	0.257	0.395 <sup>a</sup>	1	0.544 <sup>b</sup>
$\Delta$ STAI-Y2	-0.533 <sup>b</sup>	-0.654 <sup>b</sup>	0.539 <sup>b</sup>	0.603 <sup>b</sup>	0.544 <sup>b</sup>	1

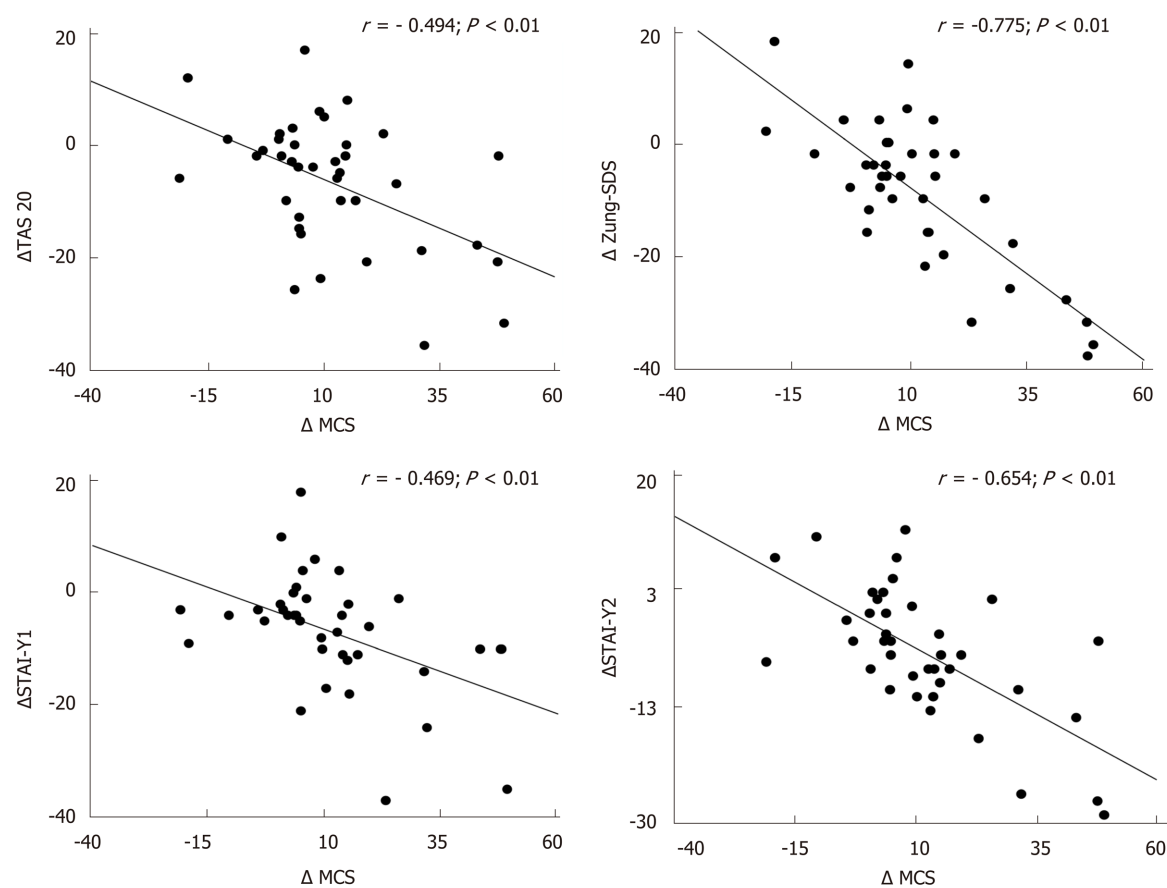
<sup>a</sup> $P < 0.05$ ;<sup>b</sup> $P < 0.01$ . DAAs: Directly acting antivirals; Zung-SDS: Zung-Self Depression-Rating-Scale; STAI-Y1: Spielberg State-Trait Anxiety Inventory Y1; STAI-Y2: Spielberg State-Trait Anxiety Inventory Y2; TAS-20: Toronto-Alexithymia Scale-20; SF-36: Short Form 36; PCS: Physical component summary; MCS: Mental component summary.**Table 7 Covariates related to the physical and mental component summary of the Short-Form-36 and to their variation before and after directly acting antivirals treatment, according to the results of the multiple linear regression analysis**

	Beta	SE (Beta)	T value	P value
Physical component summary				
Zung-SDS	-1.99	0.37	-5.38	< 0.001
Mental component summary				
Zung-SDS	-1.53	0.33	-4.62	< 0.001
$\Delta$ Physical component summary				
$\Delta$ Zung-SDS	-1.58	0.32	-4.86	< 0.001
$\Delta$ Mental component summary				
$\Delta$ Zung-SDS	-1.49	0.29	-4.98	< 0.001

SDS: Self Depression Rating Scale.



**Figure 2** Pearson's correlations between each neuropsychological test and mental component summary of Short-Form-36 at basal evaluation. MCS: Mental component summary.



**Figure 3** Pearson's correlations between the variations of Zung-Self Depression Rating Scale, Toronto-Alexithymia Scale-20, Spielberg State-Trait Anxiety Inventory Y1 and Spielberg State-Trait Anxiety Inventory Y2 and the variation of mental component summary before and after Hepatitis C virus eradication. TAS-20: Toronto-Alexithymia Scale-20; Zung-SDS: Zung-Self Depression Rating Scale; MCS: Mental component summary; STAI-Y1: Spielberg State-Trait Anxiety Inventory Y1; STAI-Y2: Spielberg state-Trait Anxiety Inventory Y2.

## ARTICLE HIGHLIGHTS

### Research background

In patients with hepatitis C virus (HCV) infection, alterations in health-related quality of life (HRQoL) and neuropsychological disturbances were described also in the absence of liver cirrhosis.

### Research motivation

During the last years, HCV therapy has evolved from interferon-based to directly acting antiviral (DAA)-based therapy, with excellent tolerability and efficacy.

### Research objectives

No data exists on the modifications of neuropsychological symptoms before and after DAAs treatment and on the relationship of these symptoms on HRQoL.

### Research methods

All patients included in the study underwent a neuropsychological assessment, including Zung-Self Depression-Rating-Scale, Spielberg State-Trait Anxiety Inventory Y1-Y2 and the Toronto-Alexithymia Scale-20 items before and after DAAs treatment. HRQoL was detected by Short-Form-36.

### Research results

In this study we demonstrated for the first time that HCV eradication strongly improves depression, anxiety and alexithymia symptoms and HRQoL.

### Research conclusions

HCV eradication is important not only in patients with liver cirrhosis, but also in patients with chronic hepatitis because significantly improves health related quality of life and neuropsychological symptoms.

### Research perspective

Further studies are needed to confirm improvements in HRQoL and neuropsychological symptoms even after years of DAAs treatment.

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## Prospective Study

# Significance of postoperative follow-up of patients with metastatic colorectal cancer using circulating tumor DNA

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## Abstract

### BACKGROUND

One of the most notable applications for circulating tumor DNA (ctDNA) detection in peripheral blood of patients with metastatic colorectal cancer (mCRC) is a long-term postoperative follow-up. Sometimes referred to as a “liquid (re)biopsy” it is a minimally invasive procedure and can be performed repeatedly at relatively short intervals (months or even weeks). The presence of the disease and the actual extent of the tumor burden (tumor mass) within the patient’s body can be monitored. This is of particular importance, especially when evaluating radicality of surgical treatment as well as for early detection of disease progression or recurrence.

### AIM

To confirm the radicality of surgery using ctDNA and compare available methods for detection of recurrence in metastatic colorectal cancer.

### METHODS

study enrollment.

**Conflict-of-interest statement:** All authors declare no conflict of interest.

**Data sharing statement:** Technical and clinical data is available from the corresponding author at lbenesova@genomac.cz. Participants have consented to use of their data for further research and other non-commercial purposes.

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A total of 47 patients with detected ctDNA and indications for resection of mCRC were enrolled in the multicenter study involving three surgical centers. Standard postoperative follow-ups using imaging techniques and the determination of tumor markers were supplemented by ctDNA sampling. In addition to the baseline ctDNA testing prior to surgery, a postoperative observation was conducted by evaluating ctDNA presence up to a week after surgery and subsequently at approximately three-month intervals. The presence of ctDNA was correlated with radicality of surgical treatment and the actual clinical status of the patient.

## RESULTS

Among the monitored patients, the R0 (curative) resection correlated with postoperative ctDNA negativity in 26 out of 28 cases of surgical procedures (26/28, 93%). In the remaining cases of R0 surgeries that displayed ctDNA, both patients were diagnosed with a recurrence of the disease after 6 months. In 7 patients who underwent an R1 resection, 4 ctDNA positivities (4/7, 57%) were detected after surgery and associated with the confirmation of early disease recurrence (after 3 to 7 months). All 15 patients (15/15, 100%) undergoing R2 resection remained constantly ctDNA positive during the entire follow-up period. In 22 cases of recurrence, ctDNA positivity was detected 22 times (22/22, 100%) compared to 16 positives (16/22, 73%) by imaging methods and 15 cases (15/22, 68%) of elevated tumor markers.

## CONCLUSION

ctDNA detection in patients with mCRC is a viable tool for early detection of disease recurrence as well as for confirmation of the radicality of surgical treatment.

**Key words:** Circulating tumor DNA; Metastatic colorectal cancer; Postoperative; Radicality of resection; Follow-up; Recurrence

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**Core tip:** Circulating tumor DNA has shown itself to be a highly specific and sensitive tool for confirming the radicality of surgical treatment in patients with metastatic colorectal cancer and for the potential prediction of early disease recurrence after R0/R1 surgeries. Additionally, when compared to imaging methods and tumor markers, circulating tumor DNA more accurately indicates disease recurrence during follow-ups that are minimally invasive and are of low burden to the patient.

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## INTRODUCTION

Around 50% of patients with colorectal cancer (CRC) are found to have synchronous liver or extrahepatic metastases at the time of diagnosis or will develop metachronous metastases within several years after surgery<sup>[1]</sup>. It is known that radical surgical resection – the R0 resection which involves a complete removal of the diseased tissue – is the only effective treatment option, ideally in combination with perioperative chemotherapy<sup>[2]</sup>. The 5-year survival rate for patients with surgical treatment ranges from 41% to over 70% compared to 5% if not treated<sup>[3-5]</sup>.

Besides removal of metastases, postoperative patient follow-up is no less important as it allows for the timely identification of any progression or recurrence of the disease and prompt therapeutical response – whether by surgery or modification of systematic therapy. The monitoring of patients with metastatic CRC is predominantly



based on imaging techniques such as ultrasonography (US), computed tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI) or X-ray imaging (XRAY), usually with assessments of serum tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). Nowadays, molecular biology techniques also play an irreplaceable role in the management of patients with CRC, considering that the decisions on cancer therapy are ever more supported by the knowledge of biological characteristics and genetic profile of the disease<sup>[6]</sup>. Most recently, the possibility for disease follow-up using circulating tumor DNA (ctDNA) has become available in addition to already implemented mutation profiling from tissue biopsies or resectates<sup>[7,8]</sup>.

The term ctDNA means short fragmented DNA, which has historically been observed in blood circulation of patients suffering with metastatic stages of cancers<sup>[9,10]</sup>. ctDNA is often referred to as cell-free DNA to emphasize its exogenous nature in comparison to DNA originating from nuclei of the blood cells. Although the exact mechanism of its release into circulation is still subject to debate, there are several probable mechanisms of the ctDNA origin including: Apoptosis; necrosis; active release through lipoprotein-nucleotide complexes (viroosomes); phagocytosis; and exocytosis<sup>[11]</sup>. Due to its exclusive origin in cancerous cells, ctDNA retains the fundamental imprint of its cancer genome including cancer-specific aberrations such as somatic mutations. Therefore, the ctDNA analysis has recently been promoted using the term “liquid biopsy”<sup>[12]</sup>. The main advantage of liquid biopsy, compared to the “classic” biopsy of tumor tissues is its minimal invasiveness and the associated minimal stress for the patient. Additionally, ctDNA is released to the bloodstream from all tumor foci, and thus a single blood sample contains the complete mutation spectrum of cancer clones present in the patient’s body<sup>[13]</sup>.

Besides liquid biopsy, ctDNA testing has proven to be a very promising instrument for long-term, postoperative follow-ups of patients with CRC, particularly in advanced stages. Being minimally invasive, it can be repeated over short periods for a long time with no significant burden to the patient, and moreover, this test shows high sensitivity and specificity to patients with preoperative ctDNA positivity<sup>[14]</sup>. The approach fundamentally relies on the applied methodology, which must be able to capture < 0.1% of ctDNA on the background of non-tumor DNA. Accordingly, the current techniques include dedicated approaches such as those based on digital PCR, BEAMing or deep sequencing<sup>[15]</sup>. In all cases, tumor-specific DNA alterations (usually mutations) found in the tumor tissue or occurring with a certain significant frequency in the given cancer are used as ctDNA positivity markers. However, the above mentioned methods have their limitations, particularly in terms of higher demands on the input DNA amount and the costs. Another sensitive, efficient, rapid and affordable method based on the formation of heteroduplexes with subsequent detection using denaturing capillary electrophoresis (DCE) has been used at our center for 10 years. This method can be used to detect a mutated locus in plasma with the sensitivity of 0.03% to 1% depending on the mutation to be determined (Table 1) with input DNA amount of tens of pg<sup>[16,17]</sup>. The specificity of this ctDNA test is 100%, which means that the presence of ctDNA always provides evidence that a tumor residue or tumor cells are present. As previously shown by us, ctDNA testing using DCE allows patient follow-up in real time, while ctDNA levels correlate very well with the current condition of the patient<sup>[17]</sup>. As we reported in this paper about a group of patients with metastatic CRC, ctDNA can be used in clinical practice, particularly for the evaluation of the radicality of surgery and for the timely detection of any progression or recurrence of the disease.

## MATERIALS AND METHODS

### *Patients and samples*

This study was conducted in cooperation with three prime surgery centers in the Czech Republic in accordance with the Declaration of Helsinki and was approved by ethics committees of the relevant hospitals. The forty-seven patients enrolled in the study had undergone the resection of synchronous or metachronous colorectal cancer metastases and tested positive for ctDNA before surgery, and were thus suitable candidates for postoperative ctDNA follow-up. Patient characteristics are listed in Table 2. All patients signed an informed consent form for the study. A tumor tissue sample was surgically obtained in each case. Peripheral blood samples for ctDNA analysis were collected in an anticoagulant solution before, 1 week after the surgery, and subsequently in several-month intervals during their follow-ups. Clinical patient data including tumor localization and type, surgical radicality, and CEA and CA 19-9 marker levels were also collected.

**Table 1** Characteristics of the markers used for the mutation analysis<sup>[16,17]</sup>

Marker	Exon number	Target codons	Size of PCR product (bp)	LOD (%)	DCE separation temperature (°C)
KRAS	2	12, 13	112	0.03	50
	3	59, 61	100	0.05	51
	4	117, 146	150	0.05	45
TP53	5	170-187	107	0.1	58
	6	187-224	169	0.5	52
	7	225-261	160	0.5	52
	8	262-307	151	0.03	56
APC	15	854-896	128	0.7	48
	15	1275-1308	100	0.7	48
	15	1290-1335	136	0.6	52
	15	1389-1446	174	0.3	48
	15	1430-1463	101	1	48
	15	1479-1530	156	1	51
	15	1539-1585	141	0.8	52
PIK3CA	9	542	106	0.2	48
	20	1025, 1031, 1047	136	0.3	49
BRAF	15	600	230	0.05	48
CTNNB1	3	45	152	0.4	52

bp: Base pair; DCE: Denaturing capillary electrophoresis; LOD: Limit of detection.

### DNA isolation and detection of mutations

Mutation profiles were determined in DNA isolated from tumor tissues, subsequently, the detected mutations were analyzed in plasma samples. Tumor DNA was isolated from the samples of native frozen (-20 °C) tissues using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich, St. Louis, MS, United States). ctDNA was isolated using the NucleoSpin Plasma XS (Macherey-Nagel, Dueren, Germany) from plasma according to the instructions of the manufacturer. Plasma was obtained by centrifugation of blood promptly after collection, and then immediately frozen at -20 °C. The mutations were identified using a panel for the detection of the most commonly mutated loci in the *KRAS*, *TP53*, *APC*, *PIK3CA*, *BRAF* and *CTNNB1* genes, which are characteristic for colorectal cancer (see Table 1 for details). Tumor tissue and ctDNA mutations were detected using PCR with the formation of heteroduplexes and subsequent separation using DCE. The principle of the method has been described previously<sup>[18]</sup>.

### Resection radicality and recurrence of the disease

The radicality of surgical removal of colorectal cancer metastases was determined by the pathologist based on examination of the resected samples' margins. Complete resections with no macroscopic or microscopic tumor residues were identified as R0. For R0 resections, the minimum distance of tumor cells from the resection line was > 0 mm. Microscopically incomplete resections, with a presence of tumor cells in the margin detected by the pathologist, were identified as R1. Macroscopically incomplete resections were identified as R2 and were classified as R2a (macroscopic presence of a residue of the primary tumor), R2b (macroscopic presence of distal metastases), R2c (macroscopic presence of any residue(s) of the primary tumor as well as of distal metastases)<sup>[19]</sup>. The surgical radicality assessments were supplemented with an analysis of postoperative ctDNA.

Disease recurrence was longitudinally monitored and was assessed using imaging methods (most commonly CT, MRI, US, XRAY) and using CEA (normal levels ≤ 5 ng/mL) and CA19-9 (normal levels ≤ 37 U/mL) tumor marker levels. Any abnormalities detected by imaging techniques or elevated levels of at least one tumor marker were considered recurrences. This data were correlated with the presence of ctDNA. The ctDNA was evaluated until recurrence or during the patient's follow-up period.

**Table 2 Clinical patient data**

Characteristics		Value
Number of patients		47
Age	mean, range (yr)	63.6 ± 12.3, 32-87
Gender	Female/male	16/31
Localization of primary	Rectum	13
	Colon	14
	Rectosigmoid	8
	Sigmoid	7
	Cecum	3
	Others	2
Localization of metastasis	Liver only	35
	Liver and/or other	12
Surgical treatment		
Number of surgeries <sup>1</sup>		63
Synchronous mCRC	Combined (primary and liver)	12
	Primary before metastases	8
	Liver first	2
	Liver in the second stage	14
Metachronous mCRC	Liver	24
	Other metastases	3
Radicality	R0	40
	R1	7
	R2	16
Recurrence (R0 surgeries)	Number <sup>2</sup>	27
	Mean time to recurrence, range (mo)	9.0 ± 5.1, 3-22

<sup>1</sup>Postoperative ctDNA was available for 50 surgeries.

<sup>2</sup>All parameters for recurrence evaluation were available for 22 R0 surgeries. mCRC: Metastatic colorectal cancer; R0: Complete resection; R1: Microscopically incomplete resection; R2: Macroscopically incomplete resection.

## RESULTS

Detailed information on the 47 patients enrolled in the study is available in [Supplementary Table 1](#). In total, 79 tumor tissue samples (25 from the primary tumor, 6 from lymph nodes and 48 from distal metastases) and 202 plasma samples (51 before surgery, 39 after surgery and 112 during follow-up) were collected over the course of the study. A detailed overview of the collected samples and mutations detected in tumor tissue is provided in [Supplementary Table 2](#).

Sixty-three resections were done during the study (including repeated resections during follow-up), which comprised 40 R0, 7 R1 and 16 R2 resections. A postoperative ctDNA sample to assess the correlation of ctDNA and surgery radicality was available for 39 patients undergoing 50 surgeries (28 R0, 7 R1 and 15 R2) ([Table 3](#); for detailed information see [Supplementary Table 1](#)). Among the 28 cases of R0 resection, postoperative ctDNA tested negative in 26 cases (26/28, 93%). After the first of the two remaining R0 surgeries, a RFA of a small metastasis in the liver, the patient continued displaying ctDNA and, subsequently, was diagnosed with disease recurrence within the scar 6 mo after the surgery. The second case of ctDNA positivity after R0 surgery was a right-sided hemicolectomy with metastasectomy. Also, in this case, the recurrence (two new liver metastases) was visible *via* imaging methods 6 months after the surgery.

In 7 cases with R1 resection, postoperative ctDNA was positive 4 times (4/7, 57%) with disease recurrence 3, 3, 4 and 7 mo after surgery in these cases. For the remaining 3 patients with negative ctDNA after R1 surgery (3/7, 43%), two of them had postoperative recurrence after 7 or 22 mo, and the third one has gone 5 months with no recurrence after surgery. Postoperative ctDNA was positive in all 15 patients with R2 resection (15/15, 100%).

Postoperative tumor recurrence was evaluated according to positive results of ctDNA testing, imaging methods and tumor marker assessments. In our set of 32

**Table 3 Correlation of surgical radicality and postoperative circulating tumor DNA**

	Number	State (time to recurrence or follow-up time)
R0	28	
ctDNA positive	2	2 recurrence (6 mo)
ctDNA negative	26	10 no recurrence (6-36 mo), 14 recurrence (4-22 mo) <sup>1</sup>
R1	7	
ctDNA positive	4	4 recurrence (3-7 mo)
ctDNA negative	3	1 no recurrence (5 mo), 2 recurrence (7 or 22 mo)
R2	15	
ctDNA positive	15	12/2/1 metastasis/primary tumor/both present
ctDNA negative	0	-

<sup>1</sup>In two ctDNA negative R0 surgeries were missing follow-up information. ctDNA: Circulating tumor DNA; R0: Complete resection; R1: Microscopically incomplete resection; R2: Macroscopically incomplete resection.

patients undergoing R0 resections of liver metastases and long-term postoperative monitoring, 22 patients had recurrences (22/32, 69%, see [Supplementary Table 1](#)). In 17 patients, from whom all parameters for the detection of postoperative tumor recurrence were available, a total of 22 recurrences were confirmed (3 patients had repeated recurrences - 2 and 4 recurrences). Thirteen times (13/22, 59%) the recurrence was simultaneously detected by ctDNA, imaging methods and tumor markers. Seven times (7/22, 32%) the tumor markers were negative and 6 times (6/22, 27%) the imaging methods were without evidence of tumor. The ctDNA was not negative even once (0/22, 0%), making ctDNA in our study the most sensitive tool for detecting tumor recurrence of all three methods. In four patients (4/22, 18%) the tumor recurrence was detected by ctDNA only, while imaging methods and tumor markers were negative ([Table 4](#)).

## DISCUSSION

Radicality of surgery is normally assessed based on histological examination of a resected sample and based on the results of imaging methods. However, these examinations cannot indicate any potential presence of circulating tumor cells found in the bloodstream or lymphatic pathways due to a metastatic process or dissemination during surgery. Potential presence of microscopic metastases cannot be demonstrated using histology or imaging methods either. Although the follow-up of tumor marker levels may be useful for detection, their sensitivity and specificity are low<sup>[20]</sup>. ctDNA is a highly dynamic marker with an approximate half-life of 2 hours, and its levels correspond to the clinical condition of patients with mCRC<sup>[17,21]</sup>. As we have shown in this paper, a postoperative presence of ctDNA correlates very well with surgery radicality, and its elevated levels in R0 and R1 resections may signal the occurrence of micrometastases and thus help to identify patients with an increased risk of early recurrence for a higher frequency of their follow-up assessments or for an indication of adjuvant therapy<sup>[22-24]</sup>.

To date, only three papers have been published studying the presence of ctDNA immediately after CRC resection. One study in 2005 focused on *KRAS* mutation persistence in the plasma of patients with colorectal cancer in various stages 3 days from radical surgery. As surprisingly indicated by the results, most patients with preoperatively detected *KRAS* mutations in plasma had the same mutations detected in plasma, also 3 days from radical surgery (7/9, 78%). Only two patients had no mutation detected in the postoperative period (2/9, 22%)<sup>[25]</sup>. As mentioned in another study, ctDNA can provide information on radicality of primary resection of colorectal cancer, which was illustrated in the case of a patient where evidence of insufficient radicality of the primary surgery was provided based on the presence of postoperative ctDNA<sup>[26]</sup>. The last study from 2016 showed a correlation between R0 resections and postoperative ctDNA negativity in 75% of patients (6/8) and a correlation between R2 resection and postoperative ctDNA positivity in 67% of patients (2/3), by presenting a set of 11 patients with preoperatively positive ctDNA results, undergoing R0 (8 times) or R2 (3 times) resection<sup>[27]</sup>.

Compared to the above mentioned studies, our study presents the largest group of patients so far with preoperatively detected ctDNA (47 in total) in whom postoperative ctDNA correlation with surgery radicality was followed, and this study

**Table 4 Comparison of circulating tumor DNA and standard detection methods**

Number of recurrences	ctDNA	Imaging methods	CA19-9 and/or CEA markers
13	+	+	+
3	+	+	-
2	+	-	+
4	+	-	-

Plus and minus symbols indicate positive and negative results of screening for recurrence, respectively. CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; ctDNA: Circulating tumor DNA.

is also the first to report ctDNA correlation with R1 resection. In our group of patients who underwent R1 resections, more than half of them were postoperative ctDNA positive. Considering that in these patients, only a small number of tumor cells were present in the resection margin that they are unlikely to release their DNA into the bloodstream, ctDNA positivity could indicate that there are still other micrometastases not detected by imaging methods. This corresponds to significantly shorter times without recurrence (3, 3, 4 and 7 mo) compared to patients with negative postoperative ctDNA (7 and 22 mo). The presence of micrometastases was also probably recognized in two postoperative ctDNA-positive patients undergoing R0 resection, as their time to recurrence was also very short (6 mo both) when compared to patients with negative R0 postoperative ctDNA (14 times recurrence after 4-22 mo, 10 times no recurrence in surveillance 6-36 mo). Unlike other studies, postoperative ctDNA positivity was captured in all R2 resections (15/15, 100%) in our study, which may be due to the sensitivity of the used detection methods. Our DCE method provides the limit of detection (LOD) of 0.03%-1% (see Table 1), while the studies referred to above used the Temperature Gradient Gel Electrophoresis method with LOD 1%-5%<sup>[28]</sup> and the Quantitative Polymerase Chain Reaction assay based on allele-specific PCR with LOD 1%-2%<sup>[29]</sup>.

Liver metastatic recurrence occurs in approximately 50% of patients undergoing hepatectomy, while 12% have a recurrence more than once. Considering that repeated hepatectomy combined with systemic therapy may improve overall survival of these patients<sup>[30-32]</sup>, efficient follow-ups for the early detection of recurrence is very important<sup>[33]</sup>.

Tumor markers are commonly used for follow-ups, but so far cannot be used alone to diagnose recurrence, and imaging methods sometimes do not recognize small foci (micrometastases) or, on the contrary, find abnormalities that are not cancer. Examination of ctDNA could be another tool used during follow-up. Our detection method enables high reliability testing (the false positive probability of ctDNA is 0%). In our 22 cases of postoperative tumor recurrence imaging methods and tumor marker results detected a recurrence in 16/22 (73%) and 15/22 (68%) cases, respectively, ctDNA was present in 22/22 (100%) cases. Our results are consistent with the findings of several similar studies that have been performed on a comparable or smaller number of samples<sup>[24,26,34-36]</sup>. Therefore, we consider our set of 22 recurrences after curative (R0) surgical treatment recorded in our study as adequate to confirm utility of the ctDNA test as a viable tool for the early detection of mCRC recurrence.

Compared to our overall ctDNA detection rate in patients with advanced CRC at the time of the diagnosis (55%-75%), the detection rate in patients previously tested positive for ctDNA is much higher. This data indicates that although the ctDNA release rate into the bloodstream due to the presence of tumor foci differs in various patients with mCRC, for each given patient this phenomenon is relatively stable; thus if ctDNA is detectable in the body when tumor foci are present, it is highly probable that after their complete removal and subsequent recurrence, ctDNA will be detected again.

Although ctDNA detection cannot replace traditional methods used in the follow-up scheme, it might be a useful supplementary instrument for both the prediction and earlier detection of recurrences, particularly in patients with a higher risk of liver metastatic recurrence<sup>[37]</sup>, and thus it may actually improve the overall survival odds of such patients.

## ARTICLE HIGHLIGHTS

### Research background

Around 50% of patients with colorectal cancer (CRC) are found to have synchronous liver or



extrahepatic metastases at the time of diagnosis, or will develop metachronous metastases within several years after surgery. It is known that radical surgical resection, the R0 resection which involves a complete removal of the diseased tissue, is the only effective treatment option, ideally in combination with perioperative chemotherapy. Besides removal of colorectal cancer metastases, postoperative patient follow-up is no less important as it allows for the timely identification of any progression or recurrence of the disease and prompt therapeutical response. The monitoring of patients is predominantly based on imaging techniques, usually with assessments of serum tumor markers. One of the promising tools for long-term postoperative follow-up is the detection of circulating tumor DNA (ctDNA) in the peripheral blood. Sometimes referred to as a "liquid (re)biopsy" it is a minimally invasive procedure and can be performed repeatedly at relatively short intervals (months or even weeks). The presence of the disease and the actual extent of the tumor burden (tumor mass) within the patient's body can be monitored. This is of particular importance, especially when evaluating radicality of surgical treatment as well as for early detection of disease progression or recurrence.

### Research motivation

Radicality of surgery is normally assessed using histological examination of a resected sample and based on the results of imaging methods. However, these examinations cannot indicate any potential presence of circulating tumor cells or microscopic metastases. Also, the main tools used for postoperative patient follow-up, imaging methods and tumor markers, are often not sufficient in early detection of disease progression or recurrence. Tumor markers have low specificity and sensitivity so that they cannot be used alone to diagnose recurrence. Imaging methods are known to fail to detect small foci (micrometastases) or, on the contrary, find abnormalities that are not cancer. Moreover, they cannot be performed frequently due to the radiation exposure. The recently introduced ctDNA testing could present a useful alternative tool. It has proven to be very promising for long-term, postoperative follow-ups of patients with CRC, particularly in advanced stages. Being minimally invasive, it can be repeated frequently for a long time with no significant burden to the patient, and moreover, this test shows high sensitivity and specificity to patients with preoperative ctDNA positivity.

### Research objectives

The main objectives of the study were to confirm the radicality of surgery using ctDNA and to compare available methods for detection of recurrence in metastatic CRC (mCRC). It is important to verify whether ctDNA can be used in clinical practice, particularly for the evaluation of the radicality of surgery and for the timely detection of any progression or recurrence of the disease.

### Research methods

A total of 47 patients with detected ctDNA and indications for resection of mCRC were enrolled in the multicenter study involving three surgical centers. Standard postoperative follow-ups using imaging techniques and the determination of tumor markers were supplemented by ctDNA sampling. In addition to the baseline ctDNA testing prior to surgery, a postoperative observation was conducted by evaluating ctDNA presence up to a week after surgery and subsequently at approximately three-month intervals. The presence of ctDNA was correlated with radicality of surgical treatment and the actual clinical status of the patient. To test ctDNA, we performed a sensitive, efficient, rapid and affordable method based on the formation of heteroduplexes with subsequent detection using denaturing capillary electrophoresis (DCE). This method can be used to detect a mutated locus in plasma with the sensitivity of 0.03% to 1% depending on the mutation to be determined with input DNA amount of tens of pg. The specificity of this ctDNA test is 100%, which means that the presence of ctDNA always provides evidence that a tumor residue or tumor cells are present. As previously shown by us, ctDNA testing using DCE allows a patient follow-up in real time, while ctDNA levels correlate very well with the current condition of the patient.

### Research results

Among the monitored patients, the R0 (curative) resection correlated with postoperative ctDNA negativity in 26 out of 28 cases of surgical procedures (93%). In the remaining cases of R0 surgeries that displayed ctDNA, both patients were diagnosed with a recurrence of the disease after 6 mo. In 7 patients who underwent an R1 resection, 4 ctDNA positivities (57%) were detected after surgery and associated with the confirmation of early disease recurrence (after 3-7 mo). All 15 patients undergoing R2 resection remained constantly ctDNA positive during the entire follow-up period. In 22 cases of recurrence, ctDNA positivity was detected 22 times (100%) compared to 16 positives (73%) by imaging methods and 15 cases (68%) of elevated tumor markers.

### Research conclusions

Although ctDNA detection cannot replace traditional methods used in the follow-up scheme, it might represent a useful supplementary instrument for both the prediction and earlier detection of recurrences, particularly in patients with a higher risk of liver metastatic recurrence, and thus it may actually improve the overall survival odds of such patients. And it has also been shown that the ctDNA test is a highly specific and sensitive tool for confirming the radicality of surgical treatment and for the potential prediction of early disease recurrence after R0/R1 surgeries.

### Research perspectives

In this study, the high sensitivity of the methodology used to test ctDNA after curative mCRC



surgical treatment and also to detect recurrence of the disease was shown. However, to confirm this hypothesis, testing on a larger sample set is required. In particular, it is desirable to obtain a greater number of ctDNA positive and negative results after R1 resections that could be correlated with time to disease recurrence. Similarly, expanding the set of long-term follow-up patients using standard approaches supplemented with ctDNA sampling is warranted.

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## Pulmonary tumor thrombotic microangiopathy of hepatocellular carcinoma: A case report and review of literature

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### Abstract

#### BACKGROUND

Pulmonary tumor thrombotic microangiopathy (PTTM) is a rare condition in patients with hepatocellular carcinoma (HCC); to date, few cases have been reported. While hepatic dysfunction has been focused on the later stages of HCC, the management of symptoms in PTTM is important for supportive care of the cases. For the better understanding of PTTM in HCC, the information of our recent case and reported cases have been summarized.

#### CASE SUMMARY

A patient with HCC exhibited acute and severe respiratory failure. Radiography and computed tomography of the chest revealed the multiple metastatic tumors and a frosted glass-like shadow with no evidence of infectious pneumonia. We diagnosed his condition as acute respiratory distress syndrome caused by the lung metastases and involvement of the pulmonary vessels by tumor thrombus. Administration of prednisolone to alleviate the diffuse alveolar damages including edematous changes of alveolar wall caused by the tumor cell infiltration and ischemia showed mild improvement in his symptoms and imaging findings. An autopsy showed the typical pattern of PTTM in the lung with multiple metastases.

#### CONCLUSION

PTTM is caused by tumor thrombi in the arteries and thickening of the pulmonary arterial endothelium leading to the symptoms of dyspnea in terminal

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staged patients. Therefore, supportive management of symptoms is necessary in the cases with PTTM and hence we believe that the information presented here is of great significance for the diagnosis and management of symptoms of PTTM with HCC.

**Key words:** Pulmonary tumor thrombotic microangiopathy; Hepatocellular carcinoma; Respiratory dysfunction; Prednisolone; Supportive care; Case report

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**Core tip:** Pulmonary tumor thrombotic microangiopathy is caused by tumor thrombi in the arteries and thickening of the pulmonary arterial endothelium leading to the symptoms of dyspnea in terminal staged patients. Therefore, supportive management of symptoms is necessary in the cases with pulmonary tumor thrombotic microangiopathy, however, as the hepatic failure, bleeding, and encephalopathy have been focused in these cases with hepatocellular carcinoma and it is rare condition in the cases with hepatocellular carcinoma, only few cases have been reported. Therefore, we have reported the minute clinical and pathological information of our recent case and reviewed literatures of reported cases to date in this paper.

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## INTRODUCTION

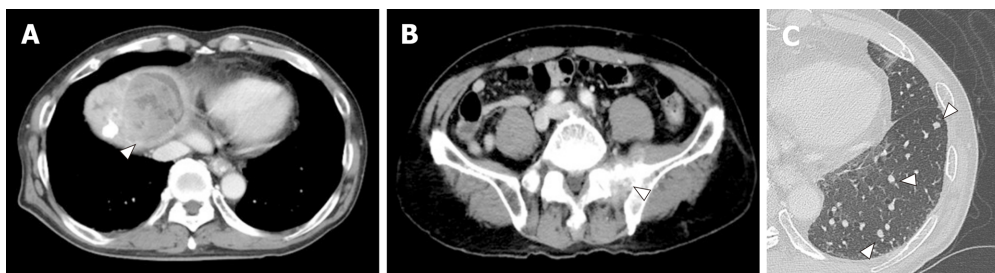
Various pathologic conditions, including diffuse alveolar lesions, lymphangiopathy, and pulmonary microembolism, are known causes of respiratory failure in cases of pulmonary malignancy<sup>[1]</sup>; however, these conditions are relatively rare in cases of hepatocellular carcinoma (HCC), possibly because HCC causes hepatic dysfunction and/or bleedings rather than respiratory dysfunction in the terminal stage. Therefore, pulmonary tumor microembolisms, including those of pulmonary tumor thrombotic microangiopathy (PTTM), are especially rare in HCC cases; and only a few cases have been reported, and the symptoms, imaging findings, therapeutic options, and prognoses have not been summarized to date. PTTM, first reported by von Herbay *et al*<sup>[2]</sup> in 1990, is a special cause of pulmonary tumor embolism in which tumor cells cause thickening of pulmonary arterial endothelium or form thrombi, which in turn cause narrowing and occlusion of the pulmonary arteries, resulting in pulmonary hypertension, dyspnea, and hypoxemia<sup>[3]</sup>. Our recent case with HCC who developed PTTM exhibited dyspnea with severe respiratory failure was diagnosed by minute histological analysis on autopsy and the information obtained was important to manage the symptoms in that stage. For a better understanding of the disease and management of symptoms, we have conducted a literature review of 18 reported cases<sup>[1,4-20]</sup> with our case.

## CASE PRESENTATION

### Chief complaints

A 72-year-old Japanese man was diagnosed with HCC and liver cirrhosis, caused by alcohol abuse, in 2011, and was referred to our hospital for therapeutic management. Since then, transcatheter arterial chemoembolization and radiofrequency ablation had been performed repeatedly, followed by the oral administration of sorafenib, 400 mg daily. After 1 year of sorafenib treatment, he was admitted to our hospital for dyspnea and low back pain. Computed tomographic (CT) scans revealed multiple HCC tumors in the liver (Figure 1A), as well as sacral bone metastases (Figure 1B) and multiple metastatic nodules in the lungs (Figure 1C) but no ascites.





**Figure 1** Computed tomographic scans of hepatocellular carcinoma. A: Computed tomographic scans of hepatocellular carcinoma (HCC) in the liver; B: Computed tomographic scans of HCC in the metastases to sacral bone; C: Computed tomographic scans of HCC in the lung. White arrowheads indicate the tumor.

### Laboratory examinations

The laboratory examination showed a mild increase in aspartate aminotransferase (74 IU/L), blood urea nitrogen (31 IU/L), creatinine (1.0 mg/dL), and C-reactive protein (5.36 mg/dL); mild decrease of prothrombin time (76% of normal), and serum albumin (3.4 g/dL). The Levels of tumor markers—alpha-fetoprotein, *Lens culinaris* agglutinin-reactive alpha-fetoprotein isoform, and des-gamma-carboxy prothrombin—were significantly increased, to 67,183 ng/mL, 37.2%, and 75,000 milli-arbitrary units per milliliter or higher, respectively (Table 1). No increase in white blood cell count, and other hepatobiliary enzymes were marked.

On the sixth day after hospital admission, the patient's respiratory condition worsened, and his blood gas analysis showed oxygen saturation (SpO<sub>2</sub>) of 91%, pH of 7.456, carbon dioxide tension of 35.2 mmHg, oxygen tension of 62.9 mmHg, bicarbonate level of 24.3 mmol/L, and BE of 0.7 mmol/L, with supplementation of 2 L/min of oxygen (Table 1, Figure 2). The chest radiograph showed a frosted glass-like shadow in the upper right lobe, middle lobe, and the lower left lobe (Figure 2). The blood and sputum cultures revealed no evidence of infectious pneumonia; however, respiratory distress and decreasing arterial blood oxygen saturation continued, and chest CT examination revealed worsening of the frosted glass-like shadow on day 8 (Figure 2). On the basis of these findings, and because antibiotics produced no response, we diagnosed his condition as acute respiratory distress syndrome, potentially a result of the lung metastasis and involvement of the pulmonary vessels by tumor thrombus.

Chest radiographs showed worsening on day 14 (Figure 2). To alleviate the respiratory failure caused by the infiltration of the inflammatory cells and the reaction in the lung, we started oral administration of prednisolone, 80 mg daily, on day 16 after admission (Figure 2). The frosted glass-like shadow on chest radiographs and CT studies (Figure 2) showed temporary improvement and the symptom of dyspnea showed mild improvement; however, the patient's respiratory condition and the data from the blood gas analysis did not improve with oxygen supplementation. The patient's general condition worsened gradually and he died on the 37<sup>th</sup> day of hospitalization (Figure 2).

With the informed consent of the patient's family, autopsy was performed to assess the cause of the respiratory failure and the frosted glass-like shadow. Macroscopically, the lung appeared to be hard and yellowish, and the presence of multiple tumors in the area was confirmed (Figure 3A). Microscopically, these tumors were confirmed to be metastases of HCC (Figure 3B), and multiple pulmonary artery tumor emboli with diffuse alveolar damages of detachment of alveolar epithelial cells, edematous changes of alveolar wall, accumulation of macrophages, and exudation of fibrinous tissue were seen (Figure 3C) and in part with recanalization in the tumor thrombus and the fibrocellular intimal proliferation (Figure 3D). In addition, medial thickening of the arterioles (Figure 3E) were seen and the tumor emboli (Figure 3F) were accompanied by CD31-positive endothelial cell growth (Figure 3G) with fibrocellular intimal proliferation (Figure 3H) which are the characteristics of PTTM.

### FINAL DIAGNOSIS

On the basis of these findings, the diagnosis was PTTM and diffuse pulmonary alveolar damage due to tumor emboli, which led to the cause of respiratory failure.

Table 1 Laboratory examination

Hematology		Biochemistry		Marker	
WBC	4840/ $\mu$ L	TP	8.0 g/dL	HBs Ag	-
Neutro	70.5 %	Alb	3.4 g/dL	Anti-HBs	-
Lymp	16.9 %	BUN	14 mg/dL	Anti-HBc	-
Eos.	3.7 %	Cre	0.59 mg/dL	Anti-HCV	-
Bas.	0.4 %	T-Bil	1.0 mg/dL		
Mon.	8.5 %	D-Bil	0.2 mg/dL	AFP	67183 ng/mL
RBC	$392 \times 10^4$ / $\mu$ L	AST	74 IU/L	AFP-L3	37.2 %
Hb	12.4 g/dL	ALT	31 IU/L	PIVKA-II	> 75000 mAU/mL
Ht.	35.9 %	ALP	828 IU/L	KL-6	300 IU/mL
Plt.	$8.0 \times 10^4$ / $\mu$ L	LDH	432 IU/L	SP-D	87.6 ng/mL
		$\gamma$ -GTP	737 IU/L		
		ChE	165 IU/L		
		NH <sub>3</sub>	92 $\mu$ L/dL		
		Na	130 mEq/L	Blood Gas Analysis of 6 <sup>th</sup> day (O <sub>2</sub> 2L)	
		K	3.8 mEq/L	SpO <sub>2</sub>	91 %
		Cl	100 mEq/L	pH	7.456
Coagulation		P	3.3 mg/dL	pCO <sub>2</sub>	35.2 mmHg
PT%	76 %	Ca	9.0 mg/dL	pO <sub>2</sub>	62.9 mmHg
PT-INR	1.15	CRP	5.37 mg/dL	HCO <sub>3</sub>	24.3 mmol/L
APTT	36.3 sec	FBS	103 mg/dL	BE	0.7 mmol/L
		HbA1c	5.5 %		
		TG	58 mg/dL		
		HDL-C	50 mg/dL		
		LDL-C	138 mg/dL		

PT: Prothrombin time activity; APTT: Activated partial thromboplastin time; BUN: Blood urea nitrogen; Cre: Creatinine; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase;  $\gamma$ -GTP:  $\gamma$ -glutamyltransferase; ChE: Cholinesterase; NH<sub>3</sub>: Ammonia; CRP: C-reactive protein; FBS: Fasting blood sugar; HbA1c: Hemoglobin A1c; TG: Triglyceride; HDL-C: High density lipoprotein; LDL-C: Low density lipoprotein; AFP:  $\alpha$ -fetoprotein; PIVKA-II: Protein induced by vitamin K absence or antagonist II; KL-6: Sialylated carbohydrate antigen; SP-D: Surfactant Protein-D; SpO<sub>2</sub>: Percutaneous oxygen saturation; BE: Base excess; HCV: Hepatitis C virus.

## TREATMENT

To alleviate the respiratory failure caused by the infiltration of the inflammatory cells and the reaction in the lung, we started oral administration of prednisolone, 80 mg daily, on day 16 after admission.

## OUTCOME AND FOLLOW-UP

The patient's general condition worsened gradually and he died on the 37<sup>th</sup> day of hospitalization.

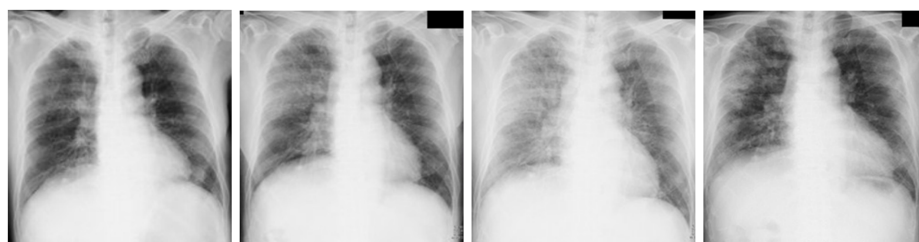
## DISCUSSION

In the cases of pulmonary microembolism caused by tumor cells, the tumor cells move intravenously or lymphatically to pulmonary arteries that are smaller than muscular arteries, and cause embolism, which may in turn cause pulmonary hypertension or respiratory failure<sup>[1]</sup>. PTTM is a special cause of pulmonary tumor embolism, in which tumor cells cause thickening of the pulmonary arterial endothelium or form thrombi, that cause narrowing and occlusion of the pulmonary arteries<sup>[2]</sup>.

In a report by Yamashita *et al*<sup>[3]</sup> who surveyed findings from autopsies of 2215 cases of malignant tumors, 30 cases (1.4%) were diagnosed with PTTM, and 21 of those



Chest X-ray



Day 6

Day 11

Day 14

Day 21

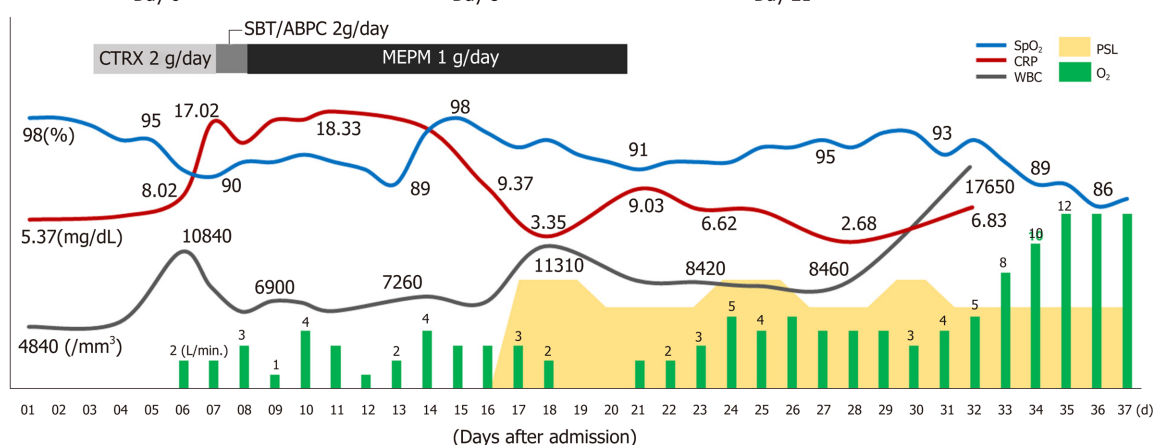
Chest CT



Day 6

Day 8

Day 21



**Figure 2** Clinical courses of physical and laboratory findings, chest radiograph, and computed tomographic scans. CT: Computed tomographic; BT: Body temperature; CRP: C-reactive protein; CTRX: Ceftriaxone sodium hydrate; MEPM: Meropenem; PSL: Prednisolone; SBT/ABPC: Sulbactam/ampicillin; SpO<sub>2</sub>: Oxygen saturation; WBC: White blood cell count.

cases exhibited hypercoagulability. Eighteen cases (60%) were with gastric cancers; the others include the carcinomas of the breast, pulmonary system, prostate, ovary, and pancreas. The most common histological type was glandular carcinoma, which was observed in 28 cases (93%). With regard to HCC as the primary lesion in cases of PTTM, only a few reports have been published to date, and the symptoms, imaging findings, therapeutic options, and prognoses have not been summarized; we performed a literature review of 18 reported cases and summarized the information with that of our case<sup>[1,4-20]</sup> (Table 2). According to our summary, the overall male-female ratio for all PTTM cases was 2:1, and of the 17 patients with PTTM that started as HCC, 15 (89%) were male (Table 2).

For the symptoms, respiratory discomfort is the chief symptom recognized with PTTM. Of the 17 patients with HCC, 9 (53%) displayed symptoms of respiratory discomfort; in addition, 4 had chest pain, 2 had pyrexia, 2 had shortness of breath, and 1 each had cough, disturbance of consciousness, and ascites. Respiratory discomfort rapidly progresses to pulmonary hypertension and right-sided heart failure, and most cases result in death a short time after the appearance of respiratory discomfort. Respiratory discomfort ultimately occurred in 13 of the 17 cases (Table 2), and of those cases, 6 (46%) presented with more than two criteria for systemic inflammatory response syndrome.

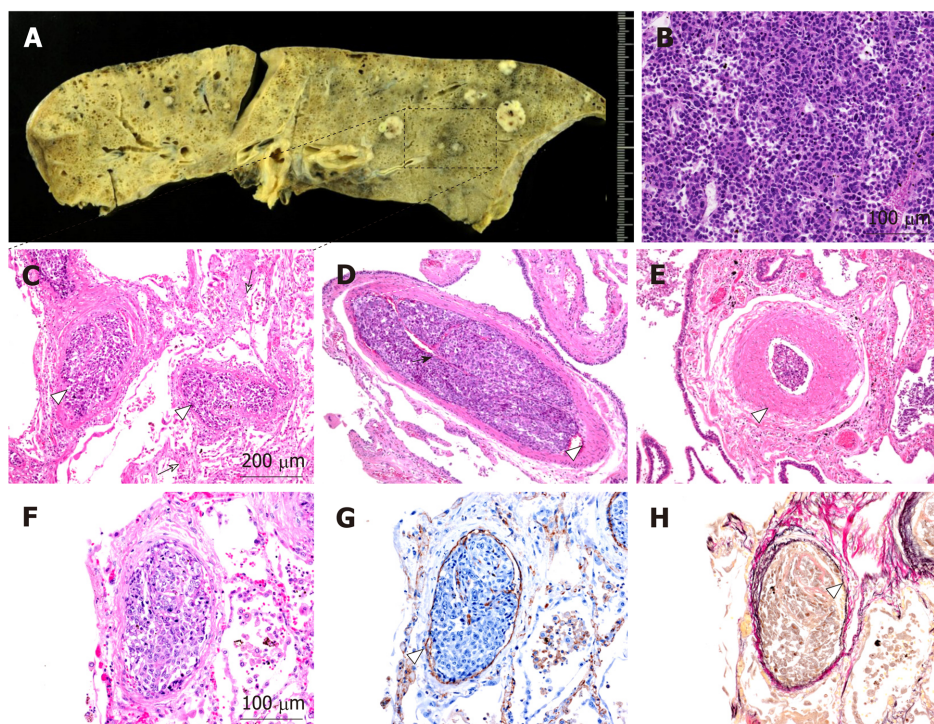
For the imaging findings of PTTM, pulmonary CT scans show consolidation which means an increase in absorption by the pulmonary parenchyma that obscures the background of blood vessels and the bronchial wall and the appearance of ground-glass attenuation, small nodules, and a tree-in-bud pattern. In our particular case, multiple small nodules appeared in the left inferior lobe; a decrease in SpO<sub>2</sub> coincided with the increase in systemic inflammatory response syndrome score; and a chest

Table 2 Summary of the cases reported

Case Ref.	Age (yr)	Gender	Etiology	Child-Pugh score	BCLC stage	Symptom	Respiratory failure	SIRS score	Invasion to IVC	Diagnosis	Treatment	Steroid	Outcome	Prognosis (d)	Image of lung	Pathology of lung	Pathology of liver
1	Uruga <i>et al</i> <sup>[1]</sup> 60	F	HCV	B	C	Dyspnea	+	2	N/A	Autopsy	Oxygen	+	Death	4	Mild elevation of CT number	Moderately differentiated HCC in lung small blood vessels	N/A
2	Nakamura <i>et al</i> <sup>[4]</sup> 52	M	Alcohol	B	C	Fever, dry cough	+	3	+	Lung scintigraphy	Decompression	+	Death	330	Multiple plaques on both lungs	Multiple tumor embolism of both pulmonary arteries	Undifferentiated HCC
3	Sato <i>et al</i> <sup>[3]</sup> 58	M	N/A	B	C	Dyspnea	+	3	N/A	Autopsy	Oxygen	-	Death	15	No imaging	Multiple pulmonary arterial tumor, thrombus	N/A
4	Shinzato <i>et al</i> <sup>[6]</sup> 56	M	N/A	N/A	C	Dyspnea, consciousness disorder	+	2	+	Autopsy	N/A	-	Death	2	Blurred nodular shadow, airbronchogram	Tumor embolism, hemorrhagic necrosis	Differentiated HCC
5	Ohta <i>et al</i> <sup>[7]</sup> 62	M	Alcohol + HCV	B	C	Chest pain	+	N/A	+	Autopsy	N/A	-	Death	60	Enhancement of pulmonary artery	Multiple pulmonary artery tumor embolism	Medium to well-differentiated HCC
6	Koskinas <i>et al</i> <sup>[8]</sup> 30	F	HBV	N/A	C	Shortness of breath	+	3	N/A	Autopsy	Oxygen	-	Death	0	No imaging	Invasion of vein by the carcinoma	N/A
7	Jäkel <i>et al</i> <sup>[9]</sup> 48	M	Alcohol	N/A	C	Ascites	N/A	N/A	+	Autopsy	N/A	-	Death	16	Unremarkable	Multiple pulmonary artery tumor embolism	N/A
8	Yamauchi <i>et al</i> <sup>[10]</sup> 58	M	HBV	N/A	C	Dyspnea	+	0	+	Autopsy	Oxygen	-	Death	5	Coin lesion	Tumor thrombi in both pulmonary arteries	Sarcomatoid HCC
9	Tanaka <i>et al</i> <sup>[11]</sup> 76	M	HCV	B	C	Dyspnea	+	N/A	N/A	Autopsy	Antibiotic, FOY	-	Death	13	consolidation in both lung field multiple defect (lung scintigraphy)	Venous thrombi of the poorly differentiated hepatocellular carcinoma	Poorly HCC
10	Nepal <i>et al</i> <sup>[12]</sup> 59	M	Alcohol + HCV	B	C	Abdominal fullness	0	1	+	N/A	N/A	N/A	N/A	N/A	Unremarkable	N/A	N/A
11	Chan <i>et al</i> <sup>[13]</sup> 52	M	HBV	N/A	C	Malaise, loss of appetite	0	N/A	+	Autopsy	N/A	N/A	N/A	N/A	No imaging	Massive necrotic tumor emboli in both pulmonary trunks.	Moderately differentiated
12	Diaz Castro <i>et al</i> <sup>[14]</sup> 71	M	HCV	N/A	C	Chest pain	+	N/A	+	Autopsy	Urokinase	-	Death	4 d	No imaging	Tumor thrombi in pulmonary arteries	N/A

13	Gutiérrez-Macias <i>et al</i> <sup>[13]</sup>	41	M	Alcohol	N/A	C	Dyspnea, chest pain, sweating	+	3	N/A	Autopsy	Antibiotic, anti-thrombotic therapy	+	Death	2	Filling defect in the left pulmonary artery	Small blood vessels occluded by clusters of malignant cells	N/A
14	Wilson <i>et al</i> <sup>[16]</sup>	65	M	N/A	N/A	C	Dyspnea	+	N/A	+	Embolus material	Antithrombotic therapy, embolic material recovery	-	Survive	N/A	No imaging	N/A	N/A
15	Mularek-Kubzdela <i>et al</i> <sup>[17]</sup>	49	M	HBV	N/A	C	Shortness of breath, lower extremity edema	+	N/A	+	CT, lung scintigraphy, United States	N/A	N/A	N/A	N/A	No imaging	N/A	N/A
16	Lin <i>et al</i> <sup>[18]</sup>	57	M	HBV	B	C	Chest pain, dyspnea	+	N/A	+	Autopsy, echocardiography	Surgery	-	Death	40	Multiple segmental perfusion defects (lung scintigraphy)	N/A	N/A
17	Papp <i>et al</i> <sup>[19]</sup>	63	M	HBV or HCV	N/A	C	Fever	-	N/A	+	Autopsy, echocardiography	Surgery	-	Death	N/A	No imaging	Tumor embolism, right atrium tumor embolism	Small round cell HCC
18	Clark <i>et al</i> <sup>[20]</sup>	65	M	HCV	N/A	C	Dyspnea, abdominal pain, malaise	+	N/A	+	Autopsy	Comfort care	-	Death	4	No imaging	The large right atrial tumor thrombus and multiple pulmonary emboli	N/A
Our case	N/A	72	M	Alcohol	A	C	Dyspnea	+	2	N/A	Autopsy	Oxygen	+	Death	37	Glass shadow of bilateral lungs	Microvascular tumor embolism in both lung	Moderate to poorly differentiated HCC

BCLC: Barcelona Clinic Liver Cancer; SIRS: Systemic inflammatory response syndrome; IVC: Inferior vena cava; HBV: Hepatitis B virus; HCV: Hepatitis C virus; N/A: Data not available; FOY: Gabexate mesylate; HCC: Hepatocellular carcinoma.



**Figure 3 Histological analyses.** A: Macroscopic findings of the lung; B: Hematoxylin and eosin staining of the tumor; C, D: Diffuse alveolar damages with multiple pulmonary artery tumor emboli. White arrows in C indicate the alveolar damage and arrowheads in C indicate tumor cells. Black arrow in D indicates the recanalization and a white arrowhead indicates the fibrocellular intimal proliferation; E: Medial thickening of arterioles. A white arrowhead indicates the thickening; F: Tumor emboli (hematoxylin and eosin staining) were accompanied by the CD31-positive endothelial cell growth; G: CD31 staining, a white arrow head and fibrocellular intimal proliferation; H: Elastica van Gieson staining, a white arrow head.

radiograph showed ground-glass opacity over the area from the right superior lobe to the inferior lobe and over to the left inferior lobe. A chest CT scan taken at the same time showed ground-glass attenuation over both lungs. The summary of the reported cases showed various imaging including tumor nodular shadows, air bronchograms, enlargement of both the heart shadow and pulmonary arterial shadows, and ground-glass attenuation, therefore, there were no specific imaging findings directly suggesting the tumor embolism or pulmonary embolism. Because there is no typical pattern in imaging findings, it is difficult to diagnose PTM while an affected patient is alive. As part of diagnosis, lung perfusion scintigraphy or cardiac ultrasonography is used to detect pulmonary hypertension<sup>[4]</sup>. In one report, cytodiagnosis was made with a specimen of pulmonary arterial blood taken with a Swan-Ganz's catheter<sup>[21]</sup>; however, this method requires caution because the procedure is highly invasive and risky in patients with respiratory distress. In that report, the patient received a definitive diagnosis but died 4 d later. Among the cases in the literature, definitive diagnosis was obtained through autopsy in 13 cases, lung perfusion scintigraphy in 2 cases, cardiac ultrasonography in 2 cases and recovery of embolus in 1 case. The pathological findings have not been described minutely, and our patient showed not only the tumor embolisms, the thickening of vascular endothelium and fiber were confirmed which are suggesting the histological features of PTM.

For therapeutic options, as far as we could confirm, all patients with respiratory distress were administered oxygen, and additional treatments included antibiotics in two cases, one case of gabexate mesilate infusion in one case, and antithrombotic urokinase therapy in two cases. No effective therapeutic options have been established for PTM at this stage. We used prednisolone infusion with the purpose of alleviating respiratory distress and improving the patient's deteriorating systemic condition, and the mild improvement of the symptom with the reduction of the ground-glass opacity in chest radiographs and CT scans were seen, however, no data of the respiratory distress and the necessary oxygen volume did not decrease. Based on the literature review, steroids were administered to three patients, and one of them showed the improvement of the images (Table 2). The prognosis for patients with PTM is extremely poor; most of such patients die within 1 week of developing respiratory distress<sup>[22]</sup>. Among the cases featured in our literature review, only one patient survived. The shortest period between the commencement of treatment for respiratory distress and death was 0, the longest was 330 d, the average was 41 d, and



the median was 9 d. PTTM is difficult to diagnose with general imaging tools, and a poor prognostic conditions with malignant tumors, therefore, the supportive care to reduce the symptoms by prednisolone, opioid, and etc. should be considered for the better terminal care.

## CONCLUSION

Our summary demonstrated the poor prognosis of the PTTM of HCC and supportive care using oxygen, prednisolone, opioid, etc. might be effective to reduce the symptoms. Further accumulation of information from cases will be of great help for physicians diagnose, manage, and care the patients and their symptoms.

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