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EDITORIAL

Percutaneous ablation of pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma is a highly aggressive tumor with an overall 5-year survival rate of less than 5%. Prognosis and treatment depend on whether the tumor is resectable or not, which mostly depends on how quickly the diagnosis is made. Chemotherapy and radiotherapy can be both used in cases of non-resectable pancreatic cancer. In cases of pancreatic neoplasm that is locally advanced, non-resectable, but non-metastatic, it is possible to apply percutaneous treatments that are able to induce tumor cytoreduction. The aim of this article will be to describe the multiple currently available treatment techniques (radiofrequency ablation, microwave ablation, cryoablation, and irreversible electroporation), their results, and their possible complications, with the aid of a literature review.

Key words: Irreversible electroporation; Pancreatic cancer; Pancreatic adenocarcinoma; Percutaneous treatment; Ablation treatment; Microwave ablation; Cryoablation; Radiofrequency ablation

Core tip: Pancreatic ductal adenocarcinoma is a highly aggressive tumor with a prognosis and treatment that depend on whether the tumor is resectable or not, which mostly depends on how quickly the diagnosis is made. In cases of non-metastatic pancreatic neoplasm that is locally advanced, it is possible to apply percutaneous treatments that are able to induce tumor cytoreduction. This article aims to expose the technical

procedures, results, and complications of the multiple treatment techniques that are currently available.

D'Onofrio M, Ciaravino V, De Robertis R, Barbi E, Salvia R, Girelli R, Paiella S, Gasparini C, Cardobi N, Bassi C. Percutaneous ablation of pancreatic cancer. *World J Gastroenterol* 2016; 22(44): 9661-9673 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9661.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9661

INTRODUCTION

Pancreatic ductal adenocarcinoma is the most common primary malignancy of the pancreas and is the most frequent exocrine pancreatic neoplasm, representing 80% of malignant pancreatic tumors[1-4].

Treatment and prognosis of pancreatic ductal adenocarcinoma depend on tumor resectability, which is usually linked with how quickly the diagnosis was made[5,6]. In almost 95% of cases, pancreatic ductal adenocarcinoma is diagnosed at an advanced stage[1], with locally advanced or metastatic disease in 40% of cases[2,6,7].

The majority of pancreatic tumors that undergo percutaneous treatment are ductal adenocarcinomas[8-11].

CLINICAL INDICATIONS

The majority of percutaneous treatment procedures, regardless of whether they involve either thermal or non-thermal ablative surgery, are dedicated to ductal adenocarcinoma[8-11] if it is locally advanced and unresectable, but not metastatic; a required indication for percutaneous treatment of ductal adenocarcinoma is therefore a locally advanced unresectable tumor, without the presence of metastases. Although tumor resectability is an absolute exclusion criterion, since complete surgical resection is the only potentially curative treatment for this tumor, only about 20% of patients present with resectable disease at the time of diagnosis. The presence of distant metastases is another exclusion criterion, and includes ascites that can be an indirect sign of peritoneal carcinomatosis[4]. It is therefore mandatory that a pathologically proved diagnosis and the correct tumor staging are obtained before percutaneous procedures.

Chemotherapy is the treatment of choice in unresectable pancreatic adenocarcinoma. Nowadays, new treatments such as radiofrequency ablation (RFA), microwave ablation (MWA), cryoablation, and irreversible electroporation (IRE) can be considered, together with chemotherapy, in multimodal therapeutic approaches for unresectable pancreatic adenocarcinoma that is locally advanced and without metastases. The palliative therapeutic effects of these treatments are intuitively linked to the development of intralesional necrosis, cytolysis, and cells death, with a resulting neoplastic mass cytoreduction[12]; some studies have explained some of the argumentations on post-ablation amplification of tumor-induced immune response[13]. These treatments can be associated with chemotherapy and could be included in a combined therapeutic plan that is tailored for each individual case[4].

TECHNIQUES AND RESULTS

There are currently many techniques for treating pancreatic ductal adenocarcinoma that is locally advanced and without metastases. These different techniques can be divided in two main groups: the thermal ablative group, which uses thermal energy, and the non-thermal ablative group, which uses energy-making direct damage to neoplastic cells that is unmediated by any thermal effect. All of these techniques can be performed in a surgery room with laparotomy or laparoscopy approaches, or they can be performed *via* percutaneous or endoscopic approaches, which are less invasive.

RFA

RFA causes coagulative necrosis and tissue damage *via* the generation of high local temperatures produced by high frequency alternating current application. RFA is currently used in many solid organs malignancies, such as hepatocellular carcinoma, thyroid neoplasms, breast and prostate tumors, and pancreatic neoplasms; it has a very important role in the treatment of hepatocellular carcinoma and has become a standard therapy[14,15].

RFA of pancreatic cancer can be guided by ultrasound both percutaneously and with endoscopic ultrasound or intraoperatively by intraoperative ultrasound; the latter is usually required in instances of biliary and gastric bypass for pancreatic head tumors[4,16,17].

The ablation procedure is performed under local anesthesia with patient sedation in both percutaneous and endoscopic approaches. In addition to the mandatory confirmation of the malignant nature of the lesion by pathological analysis before commencing treatment, it is also extremely important to carry out precise staging of the tumor.

The percutaneous approach can be more safely considered for the ablation of pancreatic tumors located in the body, as the complicated anatomy of the pancreatic head region makes the percutaneous RFA procedure more difficult and dangerous in this region. However, the pancreatic head can be treated with RFA *via* the endoscopic approach. RFA is performed with a needle that has to be inserted completely inside the lesion. The most efficient approach, needle choice, and opening degree of the electrodes are usually influenced by tumor shape and size.

There are currently two main types of needle: those with expandable electrodes (Figure 1) and those with single electrodes (Figure 2). In the former, the

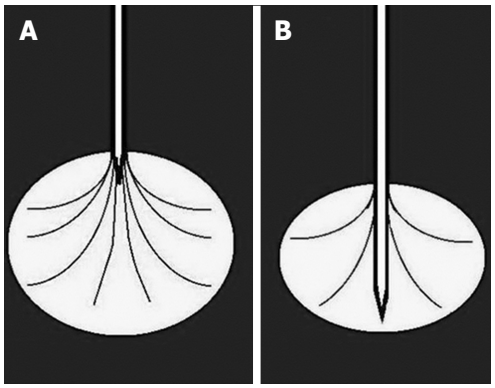

Figure 1 Needle with expandable electrodes. Electrodes can be opened within the lesion from the top (A) or from the back (B) of the needle.

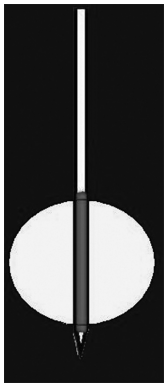

Figure 2 Needle with single electrode. Single electrode of the needle within the lesion.

expandable electrodes can be opened from the top (Figure 1A) or the back of the needle (Figure 1B), with these two needle types each being used in different way. With electrodes opened from the top, the needle tip has to be stopped immediately before the lesion and then up to nine electrodes have to be opened up and widened into the lesion. This type of needle can be used to treat small tumors because it can be opened at different degrees in relation to the size of the requested ablation area; unfortunately, however, the flexibility of electrodes can be a disadvantage in the treatment of very hard lesions. With needles where the electrodes open from back, the electrodes, usually four, are opened up at about 2 cm behind the tip and moved towards the outside so that the needle, for correct positioning, has to pass completely through the tumor; the presence of the central needle for at least 2 cm within the mass assures that the electrodes are inside the neoplastic tissue. This type of needle crosses throughout the mass with stiffer electrodes, guaranteeing excellent stability inside the tumor even in the presence of hard tumors. However, the needle's requirement to be introduced throughout the lesion for at least 2 cm can have disadvantages related to tumor location and dimensions.

Both types of expandable needle are used to produce a spherical/ovoid necrotic area, with a mean diameter ranging from 2 to 5/6 cm depending on the needle and electrode degree opening.

On the other hand, when using needles with a single incorporated electrode, the length of the area to be treated depends on the uncovered portion of the electrode, with measurement of the treated area depending on both the length of the uncovered portion and the time of ablation. This type of needle usually produces a cylindrical necrotic area ranging from 1 to 3 cm, depending on the extension of the uncovered metallic portion of the needle.

As previously stated, needle choice and the opening degree of the electrodes are usually influenced by tumor shape and size. For example it is better to use an expendable electrode needle to treat rounded lesions, while single electrode needles are preferred for ovoid lesions. The latter is also preferable in cases of very small lesions with particularly difficult access, as a needle with a lower caliper would be better in that particular situation[4].

With ultrasound it is possible to guide the ablation technique, both during the orientation of the needle and during the opening of the electrodes inside the tumor, which are both echoic. Usually the needle tip and electrodes must be kept almost 5 mm from sensitive structures such as peripancreatic vessels, as previously reported[4,18-20].

The correct needle positioning and electrode opening are followed by a procedure parameter setting that varies depending on the system used, although the procedure normally lasts from 5 to 10 min.

The power provided to the system directly influences the temperature reached and indirectly affects the treated volume of the lesion. In some cases and with some systems, it is possible to evaluate the tissue impedance that increases with necrosis growth during the procedure. The system shows the temperature at the electrode tip, which includes needles with multiple electrodes, thereby assuring a more uniform distribution of temperature in the treated lesion. Choosing the correct temperature is very important for both treatment aims and to prevent irreparable damage. As a result of the protein denaturation that starts at 50-60 ℃, higher temperatures reached during RFA lead to more homogeneous coagulative necrosis, but the use of very high temperatures (usually more than 105 ℃) increases the risk of complications without any favorable effects. For this reason, it is better to apply middle range temperatures (mean 90 ℃) to avoid complications during RFA ablation of a pancreatic mass. Several studies have confirmed that reducing RFA temperature to about 90 ℃ led to minimal complications related to the procedure[14,16,21].

The capacity of RFA to successfully treat a tumor is in part limited by two primary characteristics linked

to this heating mechanism. The first characteristic is that heating tissue with the electric current used in this process is a self-limiting process, as the desiccation, water vapor, and charring created during the RFA process gradually increase the tissue impedance that limits additional current application and limits temperatures that can be reached in the ablation area. The other characteristic is that heat transfer is moderately slow in most tissues and can differ according to local tissue environment, perfusion, and ventilation[22-24].

In a recently published paper by our institute[8], a percutaneous approach with a single tip needle with a single incorporated electrode and an exposable uncovered portion of the electrode of 17-gauge was used. In this paper, a relatively short burst of the needle is used with power set at low values (30 W) at the beginning of the procedure in order to obtain a progressive low development of coagulative necrosis and thereby allowing for intratumoral heat propagation. With respect to the uncovered portion of the needle, double necrotic volume can be obtain with this techniques thanks to intralesional passive conduction (*e.g.*, exposure of 1.5 cm can provide necrosis of more than 3 cm). This technique is reported to be particularly safe because the heat is conducted inside the neoplastic mass and not outside, which lacks conductive neoplastic tissue. This so-called "thermal diffusivity effect" is strictly related to heat neoplastic conductibility and leads to a necrotic area morphology that is quite perfectly modelled inside each single treated lesion (Figure 1). In this study, we applied a mean radiofrequency application time for every single passage of 3 min 13 s (range: from 30 s to 10 min), with 18 patients having proved unresectable pancreatic ductal adenocarcinoma that was locally advanced without metastases and were subsequently treated with percutaneous RFA. In 16 (93%) of the 18 patients, technical success of the procedure was obtained that was proven *via* a CT scan. Furthermore, one month after the treatment, a decreased blood value in CA 19.9 marker was observed in 40% of the treated patients.

Ultrasound makes possible to monitor the procedure in real-time, watching the lesion progressively becomes hyperechoic due to the gas produced inside, confirming the thermal effect obtained in the tumor, and monitoring the integrity of the sensitive surrounding structures[4,25].

Carrafiello *et al*[26] treated with a percutaneous RFA patient with pancreatic metastasis from renal cell carcinoma localized in the body-tail using a percutaneous approach under fluoro-CT guidance with deep sedation of the patient. They utilized a 19-gauge needle electrode (Invatec Miras RC, Brescia, Italy) that usually generates up to 3 cm diameter predetermined zones of necrosis. The RFA procedure lasted 8 min and 35 s and reached temperatures between 80 and 100 ℃. Although the patient reported abdominal pain, amylase serum increase, and a little peripancreatic fluid collection as complications, the result of the treatment was that he was free of symptoms without recurrence in the follow-up of 12 mo.

Limmer *et al*[27], with a CT-guided RFA, ablated an insulinoma localized in the body-tail of a patient under general anesthesia. A 16 G-cooled tip single RFA needle with 3 cm active tip was stepwise advanced into the tumor until the needle tip had reached the center of the tumor. The ablation cycle last 18 min, with the last 3 min without cooling and a maximum energy delivery that ranged from 70 to 130 W. The patient had a minimal peripancreatic inflammatory reaction and was then free of symptoms in 1.5 mo of follow-up.

Wu *et al*[28] treated, without complications, a gastrinoma in the pancreatic tail of a patient with percutaneous transplenic RFA combined with radioactive ^{125}I seed implantation. The patient was free of symptoms without recurrence in 20 mo of follow-up.

Singh *et al*[29] treated a patient with ductal adenocarcinoma *via* a CT guided percutaneous approach (the other 10 patients included in the study were treated with the surgical approach), putting the needle in the center of the tumor, and applying approximately 4200 W of energy to the tumor using a saline perfused needle with the aim of producing a necrosis of 3 cm. Partial necrosis (up to 3 cm) of the tumor was achieved in all cases. One patient died 1 mo after RFA due to massive myocardial infarction. Survival of remaining 10 patients ranged between 9 to 36 mo, with 8 patients receiving post-RFA chemotherapy. The authors concluded that RFA is a safe and feasible technique for tumor cytoreduction, but did not specify if the treated patients developed complications such as mild abdominal pain, transient ascites, self-limiting pleural effusion, or asymptomatic pseudocyst. Furthermore, they did not specify the follow-up length or medial survival.

Rossi *et al*[30] treated 8 patients under local anesthesia (7 with percutaneous RFA and 1 with endoscopic RFA) with neuroendocrine tumors (6 in the head and 2 in the body-tail; 5 with non-functional neuroendocrine tumors, 2 with insulinomas, and 1 with gastrinoma). The 19-gauge single-spiral electrode was inserted under US guidance, with the tip in the center of the tumor. The RF generator was activated to deliver 20 to 50 W for 6 to 12 min. This maneuver was repeated 1 to 4 times, depending on the tumor size. Although the same technique was used with the 17-gauge triple-spiral electrodes, a single 60-degree rotation of the shaft was sufficient to ensure full circumference ablation of the tumor. Three patients developed acute pancreatitis with fluid collections. After a median follow-up of 34 mo, all treated patients were alive and in good condition. The authors concluded that RFA is a feasible, safe, and effective option for patients with small pancreatic neuroendocrine tumors who cannot or do not wish to undergo surgical resection. A short summary of these studies is shown in Table 1.

Table 1 Summary of studies concerning radiofrequency ablation

Authors	Number of patients	Lesion type	Location	Treatment type	Approach	Needle	Mean treatment duration	Conclusion
D'Onofrio *et al*[8]	18	Pancreatic ductal adenocarcinoma	Head	Radiofrequency ablation	Percutaneous with US	17 G	3 min and 13 s	High success rate, with 40% of cases showing CA 19.9 reduction
Carrafiello *et al*[26]	1	Pancreatic metastases from renal cell	Body-tail	Radiofrequency ablation	Percutaneous with CT	19 G	8 min and 35 s	RFA is feasible for metastatic lesions at body-tail
Limmer *et al*[27]	1	Insulinoma	Body-tail	Radiofrequency ablation	Percutaneous with CT	16 G	18 min	RFA proved to be a clinically successful procedure
Wu *et al*[28]	1	Gastrinoma	Tail	Radiofrequency ablation	Percutaneous transplenic with CT	-	-	Percutaneous transplenic RFA is feasible
Singh *et al*[29]	11	Pancreatic ductal adenocarcinoma	-	Radiofrequency ablation	1 percutaneous with CT + 10 laparoscopic	-	-	RFA is a safe and feasible technique of tumor cytoreduction
Rossi *et al*[30]	8	Pancreatic neuroendocrine tumors	Head and body-tail	Radiofrequency ablation	Percutaneous with CT	17 and 19 G	9 min	RFA is a feasible, safe, and effective option

US: Ultrasound; CT: Computed tomography; RFA: Radiofrequency ablation.

MWA

Microwave radiation indicates electromagnetic spectrum frequencies ranging from 900 to 2450 MHz that lie between infrared radiation and radio waves. Electromagnetic microwaves heat material by agitating water particles in the surrounding substance, producing friction, heat, and inducing cellular death *via* coagulation necrosis[31] in a manner nearly identical to that observed in RFA[22]. In some cases, the mechanism of microwave heating may have advantages. Polar molecules (primarily water) continuously realign with the oscillating microwave field, effectively increasing kinetic energy and tissue temperature. Unlike electric currents, microwaves spread throughout biological tissues, including those with a high impedance to electric current[22,32], thereby allowing microwaves to incessantly generate heat in a larger volume of tissue around the antenna[22,33]. Microwave energy can therefore produce faster, hotter, and larger ablation areas in multiple tissues than radiofrequency current, as well as requiring fewer applicators and effective ablative margins, making it easier to obtain.

Systems that can create microwave energy are mainly divided into three types: first-generation systems that lack active antenna cooling and are limited to low power and short durations; second-generation systems with antenna cooling but limited generator power; and third-generation systems with incorporated antenna cooling and high-power generators. Each system is characterized by different combinations of antennae diameter, frequency, generated power, and power loss between the generator and applicator tip. Due to all of these features, system performance can vary a great deal, so it is therefore very important to understand that ablation zone shapes and sizes are created by different time and power associations with a particular system; MWA area volume depends on power applied, design, number of antennae, orientation of antennae, and microwave frequency.

Microwave antenna type affects the size and shape of the ablation area, with many described antennae controlling energy transfer[22,34,35]. Some antennae create an elongated ablation area of up to 6 cm called triaxial antennae, which can burn skin or other structures adjacent to the treated organ; newer antennae, such as modified triaxial antennae or dual slot antennae, therefore create more rounded and forward-weighted heating to treat smaller tumors[22,36,37]. Multiple antennae can be used together to create a larger and more confluent ablation area inside the tumor[22,38,39].

Microwaves systems also differ according to their created frequency, with the best heating typically occurring within 1 cm of the antenna. The potential advantages of MWA over RFA include faster ablations (usually 2-8 min with high-powered systems), higher temperatures without the disadvantages related to electric impedance, less sensitivity to different tissue types, and the ability to create much larger ablation zones if needed[22,32,35,40-42].

Both this ablation technique and radiofrequency ablation can be performed *via* various different approaches, including percutaneous, endoscopic, laparoscopic, and open surgical methods. Although conscious sedation of the patient is employed in the percutaneous and endoscopic approaches, the other methods use general anesthesia instead. The lesion targeted for treatment is first localized with the computed tomographic guide, although the ultrasound guide is preferred if possible, and then the best suited treatment approach is selected[31]. The tumor is then hit with a thin microwave antenna (14-15-gauge), which is in turn connected to the microwave generator with

Table 2 Summary of study concerning microwave ablation

Authors	Number of patients	Lesion	Location	Treatment type	Approach	Needle	Mean treatment duration	Conclusion
Carrafiello *et al*[47]	5	Pancreatic ductal adenocarcinoma	Head	Microwave ablation	Percutaneous with US	-	-	Microwave ablation appears to be feasible in palliative treatment

a coaxial cable and an electromagnetic microwave is released from the exposed, non-insulated portion of the antenna. Each generator is capable of producing 60 W of power at a frequency of 915 MHz. Temperatures reached inside the tumor can be measured with a separately placed thermocouple. MWA can be used in different organs, such as the liver, kidneys, lung, adrenal glands, pancreas, and bone[31,43-46].

The only paper in the literature dedicated to percutaneous MWA is that by Carrafiello *et al*[47], where 5 patients were treated percutaneously with ductal adenocarcinoma; 2 of the cases suffered from mild pancreatitis, 1 suffered from a pseudocyst, and 1 had pseudoaneurysm of the gastroduodenal artery. In all patients, MW ablation was performed under moderate sedation. The path of the antenna was carefully evaluated on the basis of a preliminary US examination, and was chosen in such a way that the vessels, stomach, and bowel were far from the antenna. The most important evaluation involves prediction of the ablation area on the basis of the antenna position. The end of the antenna features a tip such that the use of an antenna with a 37 mm radiating section results in an ablation zone with a diameter of 37 mm (7 mm on the back of the tip and 30 mm ahead of it), while the use of an antenna with a 20 mm radiating section results in an ablation zone with a diameter of 20 mm (ahead of the tip). The technical success rate was 100%, due to correct positioning of antennae. Improvement in the quality of life was observed in all patients, with none requiring repeat treatment during 9 mo follow-up. The authors concluded that patients had an improved quality of life after treatment, and that MWA appears to be feasible in the palliative treatment of cephalic pancreatic tumors, albeit with precise patient selection owing to the high risk of visceral injury. A short summary concerning this study is shown in Table 2.

Cryoablation

In the cryoablation technique, cell death is due to cold temperatures. The death mechanism in this situation differs from that under high temperatures, owing to both intracellular and extracellular ice formation during the freezing process that can result in cellular death; ice formation location and the mechanism of cell death changes with the freezing rate and final tissue temperature. Rapid freezing assists the formation of intracellular ice crystals, which result in cell death due to direct damage to the cell membrane and organelles[22,48]. On the other hand, slow freezing favors extracellular ice crystal formation, with a change of osmolality within the extracellular space consequently leading to cell dehydration and cell death[22,49]. Apoptosis induced by freezing contributes to this process, although this is a role that is poorly understood[22,50]. However, breakdown of the cell membrane with the release of intracellular contents causes cell death. Although the temperature necessary to cause necrosis depends on the cell type and the thermal history of the tissue where the lesion is, the estimated temperature is from -35 ℃ to -20 ℃[22,51,52].

During the process of cryoablation, an "ice ball" is created inside the treated area that is predictive of the ablation area. One of the greatest advantages of this technique is that this expanding ball is extremely well-visualized under ultrasound, CT, and MRI, thereby allowing for more precise monitoring of the ablation area than during heat-based techniques[22].

Systems that generate cryoablation take advantage of the Joule-Thomson theory of expanding gases within a needle-like cryoprobe: argon moves from an internal feed line into an internal expansion chamber, producing a heat sink near the antenna tip that cools the probe to temperatures of -160 ℃ or less. The heat transfer from the tissue into the cryoprobe is governed by passive thermal diffusion, so cryoablation devices provide no zone of direct or active cooling, thereby differing from RFA or MWA devices, with the result being that the surface area of the cryoprobe limits cooling efficiency; smaller diameters of cryoprobe are usually linked with lower cooling capacity and smaller ablation zones, making the use of several cryoprobes to treat the majority of the tumors indispensable in clinical practice, with high ablation times (usually mean 25-30 min) that are longer than the average times found in microwave or RFA procedures[22].

Furthermore, after the procedure there is a rapid release of cellular debris into the systemic circulation due to ablation area reperfusion, thereby sometimes causing systemic complications, such as cryoshock, that are rare with heat-based ablation[22,53].

The cryoablation technique can be performed percutaneously with ultrasound or a computed tomography guide, although it is most commonly performed intraoperatively under ultrasound guidance. As previously stated, lesions smaller than 3 cm can be treated with a single, centrally-placed probe, although larger tumors require multiple probes or sequential treatments. At 160 ℃, the tumor is cooled and the

ultrasound guide can monitor the created ice ball inside the entire lesion without compromising local structures. After ice ball creation, the tissue has to slowly thaw to 0 ℃, and then a second cycle of freezing is performed after any necessary repositioning of the cryoprobes. As with the RFA technique, it is better to have safety margins of at least 5 mm from major structures[14].

Xu *et al*[9] enrolled 49 patients with locally advanced pancreatic cancer (12 with liver metastases) that underwent cryosurgery (36 treated percutaneously and 13 treated intraoperatively) combined with ^{125}I seed implantation. The procedure was performed under local anesthesia and under the guidance of ultrasound or CT. Cryoprobe insertion was often carried out *via* the retroperitoneal approach based on the location of the tumor. Generally, 2 or 3 mm cryoprobes were used, with 2 to 3 probes being used for tumors larger than 3 cm in size (mean tumor size ranged from 2.2-7.1 cm). For liver metastases, simultaneous cryosurgery was performed using additional cryoprobes inserted through the right intercostal space. The procedure consisted of a double cycle of freeze/thaw procedure with an argon gas-based cryosurgical unit (EndoCare, Inc., CA, United States). Each cryoprobe was cooled to -160 ℃ and the resulting ice ball was monitored with ultrasound until the frozen region encompassed the entire mass of the tumor with a safety margin of at least 0.5 cm. The tissue was then allowed to slowly thaw to 0 ℃. A second cycle of freezing/thawing was performed after repositioning the cryoprobes, which were then removed. Of the 36 percutaneously treated patients, 17 received a second course of cryosurgery and 3 underwent a third. The conclusions were that cryosurgery, which is far less invasive than conventional pancreatic resection and associated with a low rate of adverse effects, should be the treatment of choice for patients with locally advanced pancreatic cancer. Furthermore, ^{125}I seed implantation can destroy the residual surviving cancer cells after cryosurgery *via* a complementary effect that combines both modalities. Complete response was observed in 20.4% of patients, partial response in 38.8% of patients, stable disease in 30.6% of patients, and progressive disease in 10.2% (5/49) of patients during a median follow-up of 18 mo (range of 5-40 mo), with a median survival duration of 16.2 mo.

Li *et al*[54] used percutaneous cryosurgery to treat 2 neuroendocrine tumors in 2 patients with MEN-1 syndrome. The first insulinoma was treated under CT guidance, where three 1.7 mm diameter cryoprobes were inserted into the tumor localized in the pancreatic tail, with 100% argon applied for 10 min. The target zone was covered by an ice ball and the cycle was repeated. The second lesion was located in the pancreatic head and preventively treated with transarterial embolization, due to its dimensions, followed by cryosurgery under ultrasound guidance. Four 2 mm diameter cryoprobes were placed into the pancreatic head tumor, frozen for 15 min and then rewarmed. All probes were extracted by 3 cm and frozen for a further 15 min. A reduction of blood glucose levels and necrosis were found under CT studies, with improvement in patient quality of life being found in the 3 mo follow-up.

Niu *et al*[55] treated 67 patients (31 with cryo-immunotherapy and 36 with cryotherapy) with ductal adenocarcinoma. Complications included abdominal distension, nausea, increased amylase serum levels, ascites, abdominal bleeding, fever, a mild decrease in blood platelet count, and an increase of fasting blood glucose levels. The obtained results showed a decreased pain score and a median overall survival that was higher after multiple cryoablations than after a single attempt. A short summary concerning these studies is shown in Table 3.

IRE

IRE is a non-thermal technique that can be used in the treatment of locally advanced non-metastatic pancreatic adenocarcinoma by inducing cell death. The ablative effect, with its consequential cell death, is based upon the use of a short high-voltage electric current. The application of these short high-voltage electric pulses, guided by one or more monopolar electrodes placed within the lesion, causes irreversible permeabilization of lipid membranes, leading to the disruption of cellular homeostasis and the switching on of apoptotic pathways, thereby resulting in the cell death of neoplastic cells[56-63]. IRE can be performed both during surgery and percutaneously.

This emerging non-thermal ablative technique uses electrodes placed within the targeted lesion in order to deliver up to 3 kV of direct current, thereby inducing the formation of nanoscale pores within the cell membrane, irreversibly damaging the cell homeostatic mechanism, and causing apoptosis[14].

One of the positive features of IRE that separates it from RFA is the ability to preserve surrounding structures, such as the underlying tissue and the vital surrounding structures such as nerves or vessels[56,64-66]. This ability of IRE of preserve vessels could be a very important aspect for evaluation in cases of tumors that encase major peripancreatic vessels.

However, this treatment is not a "pure" non-thermal technique, as it could cause partial cellular damage in a thermal manner and, in some conditions of high intensity, the current applied can produce coagulative necrosis similar to those produced by thermal techniques such as RFA or MWA[56,67]. Other studies have concluded that IRE does not produce significant thermal energy, at least when using settings most commonly applied in clinical treatment, but it has been shown that the presence of a metallic stent could increase the risk of producing thermal injuries, due to metal conductivity[56,68]. This aspect is therefore fundamental to keep in mind when treating patients

Table 3 Summary of studies concerning cryosurgery

Authors	Number of patients	Lesion	Location	Treatment type	Approach	Needle	Mean treatment duration	Conclusion
Xu *et al*[9]	49	Pancreatic ductal adenocarcinoma	-	Cryosurgery	36 percutaneous with US or CT + 13 intraoperative	2 or 3 mm	-	Cryosurgery is associated with a low rate of adverse effects
Li *et al*[54]	2	Neuroendocrine tumors	Head and tail	Cryosurgery	Percutaneous with US and CT	1.7 mm and 2 mm	10 and 15 min	Percutaneous cryosurgery is minimally invasive and has advantages compared with conventional surgery
Niu *et al*[55]	67	Pancreatic ductal adenocarcinoma	-	Cryosurgery	Percutaneous with US and CT	1.7 mm	-	Cryoimmunotherapy significantly increased overall survival in metastatic pancreatic cancer

US: Ultrasound; CT: Computed tomography.

who are carrying a biliary metallic stent for jaundice palliation.

The planning of IRE treatment has extreme importance. Several tools can be used to help select the best treatment plan and, as shown in some papers, can also accurately describe the procedure under ideal settings in cases of pancreatic tumor treatment[56,69,70].

One of the greatest restrictions of IRE is the need for general anesthesia with complete muscular paralysis[71]. Bagla *et al*[72] described a case report of percutaneous ablation of a pancreatic cancer in the body-tail, planned and performed as two ablative sessions in order to avoid the need for more than six probes to be placed at once. The treatment was performed with four 15 cm monopolar probes (NanoKnife; AngioDynamics, Latham, New York, United States) placed into the central and lateral aspects of the tumor under ultrasound guidance in a square configuration, with an average spacing of 1.8 cm. CT imaging with contrast medium was then performed to evaluate needle position relative to vessels and measure inter-probe distance. A 22-gauge spinal needle (Becton Dickinson, Franklin Lakes, New Jersey) was placed under US guidance into the gastrohepatic space to perform hydrodissection with sterile water. The treatment had no complications and patients suffered no recurrence upon a 6 mo follow-up. The authors concluded that percutaneous IRE showed promise as a feasible and potentially safe method for local tumor control in patients with surgically unresectable disease.

Martin *et al*[73] described 27 pancreatic cancer patients (15 in the head and 12 in the body-tail), of whom 26 were treated with surgical IRE and 1 with percutaneous IRE. IRE was performed *via* the NanoKnife system (AngioDynamics, Latham, New York, United States). Two monopolar probes with 2 cm spacing delivered electroporation defect of approximately 3.5 cm axial, 2.5 cm anterior-posterior, and 2.5 cm cranial-caudal. This electroporation defect was achieved through a maximum of 1.5 cm exposure, 1500 volts/cm, and with 100 microsecond wavelength. No specific complications occurred to the percutaneously-treated patient among hematologic disorders, ileus, bile leak, portal vein thrombosis, deep venous thrombosis, pulmonary infections, renal failure, ascites, and wound infection. The authors concluded that IRE ablation of locally advanced pancreatic adenocarcinoma is safe and feasible as a primary local treatment in unresectable locally advanced disease.

In a 2012 paper, Narayanan *et al*[10] treated 14 cases of ductal adenocarcinoma (6 in the head, 1 in uncinated process, and 7 in body-tail) using the NanoKnife IRE device (AngioDynamics, Latham, New York, United States). The IRE was set up to produce 70-microsecond high-voltage (1500-3000 V) direct current (25-45 A) electrical pulses. Unipolar electrodes were more commonly used, and the maximum separation between the electrodes was 2.2 cm. No tissue separation maneuvers were used to protect structures adjacent to the IRE electrodes. The electrodes were advanced percutaneously under CT guidance. Bipolar probes were positioned within and around the tumor, and current was applied when CT had confirmed adequate position. The generator was programmed to stop delivery and recharge if the current flow exceeded 48 A. Pull-back was performed if the target treatment zone was greater than 2 cm, and treatment was repeated to cover the entire target. As the only complications encountered were 1 case of pancreatitis and 1 case of pneumothorax during anesthesia, the authors concluded that percutaneous IRE for pancreatic adenocarcinoma is feasible and safe. 1 patient was retreated owing to local disease progression after 7 mo. Due to downstaging, two patients had to receive surgery. The median event-free survival after the IRE procedure was 6.7. The 6 mo overall survival was 70%.

Månsson *et al*[11] included 24 patients in their study

Table 4 Summary of studies concerning irreversible ablation

Authors	Number of patients	Lesion	Location	Treatment type	Approach	Needle	Mean treatment duration	Conclusion
Bagla *et al*[72]	1	Pancreatic ductal adenocarcinoma	Body-tail	Irreversible electroporation	Percutaneous with US and CT	22 G	-	Percutaneous IRE showed promise as a feasible and potentially safe method for unresectable tumor
Martin *et al*[73]	27	Pancreatic ductal adenocarcinoma	15 head + 12 body-tail	Irreversible electroporation	1 percutaneous + 26 surgical	-	-	IRE ablation is safe and feasible as a primary local treatment in unresectable locally advanced disease
Narayanan *et al*[10]	14	Pancreatic ductal adenocarcinoma	6 head + 1 uncinated process + 7 body-tail	Irreversible electroporation	Percutaneous with CT	-	-	Percutaneous IRE in pancreatic adenocarcinoma is feasible and safe
Månsson *et al*[11]	24	Pancreatic ductal adenocarcinoma	19 head + 5 body-tail	Irreversible electroporation	Percutaneous with US	-	-	Percutaneous IRE is reasonably safe and shows promising results for efficacy

US: Ultrasound; CT: Computed tomography; IRE: Irreversible electroporation.

who had locally advanced pancreatic cancer (19 in the head and 5 in the body-tail), prior chemotherapy and/or radiochemotherapy, and underwent percutaneous IRE. NanoKnife IRE equipment from AngioDynamics (Latham, New York, United States) was used. In all patients, the ultrasound-guided needle placement outlined the tumor, with a needle also placed in the center of the tumor when the tumor diameter exceeded 2.0 cm. The electrical parameters were calculated by the machine in order to compensate for any error in the assessment of needle distance. Six needles were used in twenty patients, four needles in two patients, and three needles in the remaining two patients. The active needle length was 1.5 cm in all cases. A minimum of 90 pulses was delivered between each adequate needle pair, which was defined as the distance between the needles not exceeding 2.5 cm. After completion of the treatment cycles in the deep portions of the tumors, the needles were pulled back 1.5 cm and another treatment with the same parameters was performed in the superficial portion of the tumor. In 11 patients, IRE-related complications were observed (infections and thrombosis), of which 3 were serious. There was no IRE-related mortality. Recurrence occurred in 9 patients. The overall survival was 17.9 mo. The authors concluded that percutaneous IRE after chemotherapy/radiochemotherapy appears reasonably safe and shows promising results with regards to efficacy. A short summary of the results is given in Table 4.

POST-OPERATIVE IMAGING AND COMPLICATIONS

The goal of all these treatments is to reduce tumor volume *via* cytoreduction. In the case of pancreatic treatment, since ductal adenocarcinoma is significantly hypovascular, detection of the created necrotic area with respect to residual viable tumor tissue can be very difficult. A very important difference between RFA of the pancreas and that of the liver is that, in the treatment of pancreatic tumors, the presence of residual viable tumor at the periphery of the treated area is almost always an intrinsic aspect of the procedure, as the necrotic area must be included in the tumor. Additionally, in other thermal ablative procedures, such as MWA and cryoablation, the ablated area has to be kept inside the tumor in order to avoid complications due to thermal energy outside the pancreatic parenchyma. The most important difference among all thermal procedures and IRE is that is possible with IRE to create necrosis throughout the tumor, as opposed to merely inside it, thereby hypothetically destroying all the cells within the lesion in order to eliminate viable tissue borders outside the induced necrosis.

To evaluate the resulting ablated area, dynamic examination after contrast agent injection is usually performed from one month after the procedure, as it is able to detect the intratumoral necrotic area produced by thermal ablation treatment. In our experience, patients treated with percutaneous RFA for pancreatic adenocarcinoma should undergo a CT scan one day after the procedure in order to completely exclude the presence of some complications. If no complications are present, patients should undergo another CT scan one month after the previous scan as part of routine follow-up. If the ablated area results under dynamic CT scan are slightly hyperdense at the basal scan, then they are obviously avascular throughout the dynamic phases, thereby maintaining the same basal density. In particular, while the arterial and venous phases are very important in highlighting vascular complications

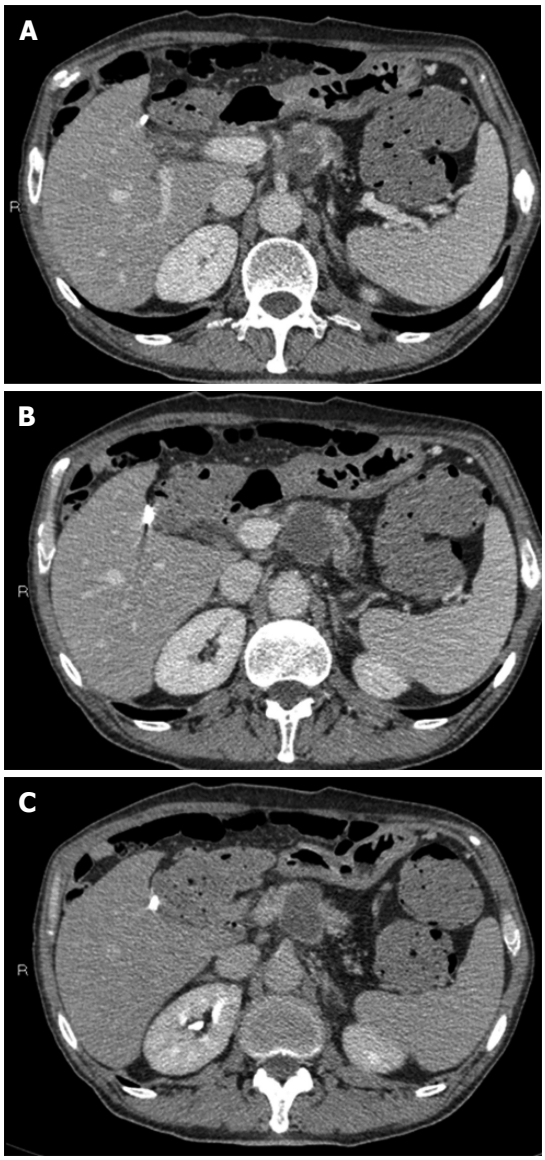

Figure 3 Radiofrequency ablation of pancreatic cancer. Computed tomography (CT) scan in the portal phase (A, B) shows the markedly hypodense necrotic avascular area modelled within the tumor. CT scan in the late phase (C) shows the ablated area as being better delineated from the enhanced adjacent tissue.

such as pseudoaneurysms and venous thrombosis, the late phase is vital for showing the ablated area as being better delineated from the enhanced adjacent tissue (Figure 3). The late phase is therefore best for measuring the ablated area.

Tumor reduction greatly increases the possibility of an improved quality of life and life expectancy, as is possible to reduce the pain caused by pancreatic tumors by causing necrosis within it. Causing cytoreduction also makes it possible to reduce the possibility of rapid disease progression[4,14,54,74-78]. Furthermore, these procedures of creating necrosis inside the tumor can reduce tumor markers and induce an immune response towards the tumor, thereby releasing factors from the tumor that are able to stimulate the immune response and activate host anti-tumor immunity in order to control micrometastases and generate tumor resistance[8,12]. More studies are necessary in order to assess the ability of these treatments to improve life expectancy, as there are currently few in the literature.

Percutaneous pancreatic cancer treatment causes minor complications, including abdominal pain, nausea, fever, skin burn, and fluid collection, as well as major complications such as a comprised splenic vein, portal vein thrombosis, digestive or abdominal bleeding, duodenal perforation, pancreatic fistula, infection, severe pancreatitis, and death.

In our experience of 18 cases of pancreatic adenocarcinoma treated with percutaneous RFA, none of the patients developed intra- or post-procedural complications[8].

CONCLUSION

Currently, ablation procedures are combined with chemotherapy for the treatment of pancreatic cancer. The percutaneous approach is one of the reported possible routes to guarantee minimal invasiveness.

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REVIEW

Fatty liver is associated with an increased risk of diabetes and cardiovascular disease - Evidence from three different disease models: NAFLD, HCV and HIV

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Abstract

Fatty liver, which frequently coexists with necro-inflammatory and fibrotic changes, may occur in the setting of nonalcoholic fatty liver disease (NAFLD) and chronic infections due to either hepatitis C virus (HCV) or human immunodeficiency virus (HIV). These three pathologic conditions are associated with an increased prevalence and incidence of cardiovascular disease (CVD) and type 2 diabetes (T2D). In this multidisciplinary clinical review, we aim to discuss the ever-expanding wealth of clinical and epidemiological evidence supporting a key role of fatty liver in the development of T2D and CVD in patients with NAFLD and in those with HCV or HIV infections. For each of these three common diseases, the epidemiological features, pathophysiologic mechanisms and clinical implications of the presence of fatty liver in predicting the risk of incident T2D and CVD are examined in depth. Collectively, the data discussed in this updated review, which follows an innovative comparative approach, further reinforce the conclusion that the presence of fatty/inflamed/fibrotic liver might be a shared important determinant for the development of T2D and CVD in patients with NAFLD, HCV or HIV. This

review may also open new avenues in the clinical and research arenas and paves the way for the planning of future, well-designed prospective and intervention studies.

Key words: Atherosclerosis; Cardiovascular risk; Fatty liver; Fibrosis; Hepatitis C-associated dysmetabolic syndrome; Hepatitis C virus; Human immunodeficiency virus; Nonalcoholic fatty liver disease; Steatohepatitis; Steatosis; Virus-associated fatty liver disease

Core tip: Normally, the liver is almost devoid of fat and fatty changes often coexist with necro-inflammatory and fibrotic changes in the setting of nonalcoholic fatty liver disease (NAFLD), chronic infection due to hepatitis C virus (HCV) or human immunodeficiency virus (HIV), which have all been associated with an increased prevalence and incidence of cardiovascular disease (CVD) and type 2 diabetes (T2D). On these grounds, in this multidisciplinary clinical review, we discuss the ever-expanding wealth of evidence supporting a key role of fatty liver in the development of T2D and CVD both in patients with NAFLD and in those with HCV or HIV infections.

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INTRODUCTION

The storage of excess lipids is not among the physiologic functions of the liver, which normally is almost devoid of fat content[1]. Fatty liver is defined as the presence of triglycerides in more than 5% of the hepatocytes. In many cases, such fatty changes (simple steatosis) may frequently coexist with lipotoxic features, such as necro-inflammation and fibrosis (*i.e.,* steatohepatitis), the extent of which depends on the etiology of such liver changes[2].

Fatty liver may occur in the setting of both metabolic and viral injuries. For example, nonalcoholic fatty liver disease (NAFLD) is defined by fatty changes which occur in individuals without excessive alcohol consumption and who have (or will develop) features of the metabolic syndrome (MetS)[3,4]. Chronic hepatitis C virus (HCV) infection is also commonly associated with fatty liver, which occurs to a variable extent according to the viral genotype and host's metabolic features[5]. Similarly, human immunodeficiency virus (HIV) infection exhibits fatty liver as a result of multiple viral and host factors, probably including the anti-viral drugs used[6]. Fatty liver associated with these two highly prevalent viral infections has collectively been named "virus-associated fatty liver disease" (VAFLD)[7].

Recent studies have addressed the systemic and cardio-metabolic risks associated with fatty liver due to different etiologies. For example, both NAFLD and VAFLD have been linked with an increased risk of cardiovascular disease (CVD) and type 2 diabetes (T2D). However, in clinical practice, different medical expertise is involved in the diagnosis and management of CVD risk; NAFLD; and HIV mono or co-infection.

In this updated clinical review, we summarize the rapidly expanding body of evidence supporting a contribution of fatty liver *per se* in the development of CVD and T2D not only in patients with NAFLD but also in those with chronic HCV or HIV infections. For each of these diseases, we extensively discuss the epidemiological burden, pathophysiologic mechanisms and clinical implications of the presence of fatty liver in predicting the risk of developing T2D and CVD. We believe that this review, which follows an innovative multidisciplinary and comparative approach, further reinforces the notion that the fatty/inflamed/fibrotic liver may represent a shared and important determinant for the development of T2D and CVD in patients with any of these three common steatogenic diseases.

NAFLD

Epidemiology, risk factors and natural history of NAFLD

The prevalence of NAFLD in the general adult population is approximately 25%-30% in Europe and United States based on imaging studies, *i.e.,* roughly two-fold higher than that identified through serum liver levels; the highest prevalence of disease is observed in Southern American and Middle Eastern countries while the lowest prevalence is found in Africa[8]. An "inverted U" shaped curve describes the prevalence of NAFLD as a function of increasing age, suggesting that younger and older individuals are relatively more spared from NAFLD[9].

The incidence rates of NAFLD have been estimated between 52/1000 person-years in Asia and 28/1000 person-years in Israel, respectively[8]. However, very little is known about the incidence rates of NAFLD in Western countries. A pioneering study conducted in northern Italy found that NAFLD incidence was approximately 2/1000 person-years in a sample of women subjected to hysterectomy[10].

The metabolic predictors of NAFLD are likely to vary based on sex[11]. Compared to women, men are at higher risk of developing NAFLD in most published studies[2,12], and post-menopausal women are no longer spared from NAFLD and its fibrotic evolution, owing to ovarian senescence and estrogen deficiency[13,14].

Table 1 Metabolic disorders associated with nonalcoholic fatty liver disease, listed in descending order

From: Younossi *et al*[8] 2016		From: Lonardo *et al*[15] 2015	
Feature	Strength of associations (% Prevalence of a given metabolic condition in those with NAFLD)	Feature	Strength of associations (% Prevalence of NAFLD in those with a given metabolic condition)
Hyperlipidemia	69%	Obesity	98%
Obesity	51%	Mixed hyperlipidemia with elevated serum ALT levels	83%
MetS	42%	T2D	70%
Hypertension	39%	Mixed hyperlipidemia	60%
T2D	22%	MetS	70%
		Hypertension	50%

ALT: Alanine transaminase; MetS: Metabolic syndrome; T2D: Type 2 diabetes.

It is known that abdominal obesity/overweight, T2D and other MetS features are strongly associated with an increased risk of NAFLD. However, the hierarchy of individual MetS features associated with NAFLD risk varies across the different studies. For example, one can report the prevalence of NAFLD in patients with MetS features as opposed to the prevalence of MetS features in patients with NAFLD (Table 1)[8,15]. Of concern, most of the MetS features listed in Table 1 are positively associated with the development and progression of NAFLD[3,16-19].

The natural history of NAFLD features both hepatic and extra-hepatic outcomes, which have been discussed in detail elsewhere[20-22]. The extra-hepatic outcomes of NAFLD include T2D/metabolic, CVD and cancer. Simple steatosis, with/without minor degrees of necro-inflammatory changes, is the first step in the histogenesis and natural history of NAFLD. Traditionally, NASH has been postulated to develop in a subset of "*bad storers*", leading to the notion that simple steatosis and NASH are two different pathologic conditions with little, if any, mutual interconnections[23,24]. However, recent studies have demonstrated that simple steatosis can progress to NASH and that both conditions progress to advanced fibrosis, though this occurs at a much slower pace for simple steatosis than for NASH[16,25-27]. The severity of hepatic fibrosis, rather than NASH, largely dictates the prognosis of liver-related outcomes in NAFLD[21,28,29].

The often used statement "NAFLD is the hepatic manifestation of the MetS" fails to render the mutual and bidirectional link existing between these two diseases. Notably, such statement also fails to pinpoint that NAFLD is a precursor and almost doubles the risk of developing T2D and MetS over a median follow-up period of 5 years[4,30].

Strong evidence also indicates that NAFLD is associated with an increased risk of subclinical atherosclerosis[31-34]. The entity of this NAFLD-related proatherogenic risk is probably linked to the severity of hepatic fibrosis[35], the degree of fatty liver[36], or both. More importantly, follow-up studies have consistently shown that CVD is the leading cause of mortality in NAFLD patients[37,38]. However, it should also be pointed out that fatal and non-fatal CVD complications are not the only dreadful outcomes in patients with NAFLD. Indeed a variety of other cardiac complications have recently been reported in these patients, including aortic-valve stenosis, cardiac arrhythmias, and increased re-hospitalization rates following discharge for acute heart failure[39-41].

Patients with NAFLD have an increased risk of developing hepatic and extra-hepatic cancers. The risk of hepatocellular carcinoma (HCC) is not only confined to patients with cirrhosis and accordingly, in NAFLD patients, HCC tends to escape those surveillance protocols which are a standard of good clinical practice in individuals with viral chronic liver diseases[42-45]. Further to HCC, increased rates of cholangiocarcinoma have been reported in NAFLD patients[46-48]. Finally, a variety of extra-hepatic cancers have also been reported in patients with NAFLD, including colo-rectal, pancreatic and uterine cancers[49], though the consistency of these associations remains to be definitely proven.

Epidemiological evidence for an association of NAFLD with T2D

The prevalence of NAFLD is remarkably increased in patients with T2D, ranging from 30% to 75% according to age, ethnicity, the study population and the diagnostic tools used[15].

Several retrospective and prospective studies have shown that NAFLD, as diagnosed either by raised serum liver enzymes or ultrasonography, independently predicts the development of incident T2D and MetS[30,50-52]. Recently, two meta-analyses concluded that NAFLD is associated with a two-fold increased risk of incident T2D and MetS[4,53]. Consistently, an improvement[52,54] or a transient remission of NAFLD significantly decrease risk of new-onset T2D[55].

To date, the only small retrospective cohort study investigating the association between biopsy-proven NAFLD and risk of incident T2D found that most of these NAFLD patients developed T2D over a 13.7-year follow-up, and that T2D risk was almost three-fold higher in patients with NASH than in those with simple

steatosis[56].

The presence of T2D also worsens the histological course of NAFLD. Patients with NAFLD and T2D have a high prevalence of NASH and advanced fibrosis[3,15,57-59], and consistently, T2D is a strong predictor of the severity and progression of histologically assessed hepatic fibrosis[25,27,57,60]. Moreover, T2D is also an important predictor of the development of HCC[61,62] and an increased risk of all-cause and liver-related mortality[60,63,64].

Worryingly, T2D patients often exhibit advanced histological disease with normal serum transaminases and may also develop HCC even in the absence of cirrhosis[44,61,65] resulting in restricted therapeutic options and decreased survival[43].

Epidemiological evidence for an association of NAFLD with CVD

A large number of studies have consistently shown that NAFLD is strongly associated with various markers of subclinical atherosclerosis, independent of traditional CVD risk factors and MetS features[31,34,66]. Epidemiological studies[37,39,67] have also shown that NAFLD is independently associated with a greater severity of coronary stenoses and an increased prevalence of carotid atherosclerotic plaques[66,68,69].

Against this background, a consistent body of evidence also suggests an association between NAFLD and increased incidence of fatal and non-fatal CVD events in NAFLD patients with/without T2D[38,39,67,70]. Indeed, as extensively reviewed elsewhere[34,39], population-based cohort studies, have reported a strong and independent association between NAFLD and increased risk of fatal and non-fatal CVD events independent of multiple cardio-metabolic risk factors[70-81]. Only one of these studies had assessed coronary artery disease (CAD) as a pre-specified outcome; it reported that patients with NAFLD had a higher 10-year risk for CAD as calculated by the Framingham risk score (FRS) than the matched control population, featuring almost the same number of FRS-predicted and actual new CAD events[76]. A recent prospective study, involving 125 patients with ultrasound-diagnosed NAFLD and 250 age- and sex-matched control individuals, followed-up for 10 years, confirmed that the incidence of CVD outcomes was significantly higher in patients with NAFLD[82]. Studies have also highlighted that middle-aged individuals with NAFLD are particularly prone to increased CVD mortality[79,83]. For example, a study enrolling 980 subjects with NAFLD (diagnosed by elevated serum aminotransferase levels) compared to 6594 NAFLD-free individuals, followed up for 8.7 years, reported that the presence of NAFLD was associated with a higher risk of all-cause and CVD mortality, independently of traditional CVD risk factors in the 45-54 age group[83]. A recent Asian cohort study performed on NAFLD patients submitted to coronary angiography paradoxically showed a reduced CVD incidence over the follow-up period; however, the successful coronary re-vascularization procedures and the intensive lipid-lowering therapy might have dramatically improved the prognosis in this specific cohort of patients[84].

Notably, several cohort studies with a reasonably long follow-up that used biopsy-proven NAFLD have clearly shown that this disease is associated with an increased risk of all-cause and cause-specific mortality (mainly CVD, cancer-related and liver-related)[28,29,56,85-87]. Most of these studies showed that the severity of hepatic fibrosis was the main determinant of all-cause and cause-specific mortality, and that CVD was the leading cause of death in patients with NAFLD[28,29,56,85-87]. Despite some studies reporting NASH more closely associated with CVD risk than simple steatosis, a milestone meta-analysis did not find any significant difference in CVD risk among patients with NASH and those with simple steatosis; nevertheless it confirmed an increased CVD risk in patients with NAFLD[53]. Recently, a systematic review and meta-analysis involving 16 observational prospective and retrospective studies with 34043 adult individuals (36.3% with NAFLD) and approximately 2600 CVD outcomes (> 70% CVD deaths) over a median period of 6.9 years found that NAFLD patients had a higher risk of fatal and non-fatal CVD events than NAFLD-free controls[88]. Patients with more "*severe*" NAFLD were also more likely to develop fatal and non-fatal CVD events[88].

Of relevance, persistent NAFLD on ultrasound also appears to be a risk factor for incident subclinical atherosclerosis, and by treating NAFLD, we may improve vascular health. In fact, a retrospective cohort study of 8020 Japanese adults found that persistent NAFLD was associated with an increased risk of developing subclinical carotid atherosclerosis, and that this association was largely explained by coexisting cardiometabolic risk factors[89]. Consistently, a small randomized clinical trial, by evaluating the effect of a 18-mo treatment with either omega-3 fatty acids or placebo on the carotid intima-media thickness (IMT) progression, found that improvement in two markers of NAFLD severity was independently associated with decreased carotid IMT progression[90].

Pathogenic mechanisms of T2D and CVD in NAFLD

T2D in NAFLD: *Via* ectopic fat storage at multiple organ sites, insulin resistance (IR) is a key pathogenic determinant of T2D development in predisposed individuals with NAFLD. In particular, IR results from storage of ectopic fat in the liver and skeletal muscles owing to long-standing excess of energy supply and subsequent infiltration of macrophages into white adipose tissue[91].

Recent research focused on the complex and bidirectional relationship between IR and NAFLD. On the one hand, IR is an established risk factor of NAFLD[3],

which occurs owing to unopposed lipogenic pathways being triggered by IR *via* multiple transcription factors, such as carbohydrate-responsive element-binding protein, liver X receptors, sterol regulatory element-binding protein 1C and upstream stimulatory factors[92]. On the other hand, NAFLD *per se* is a major determinant of hepatic IR. Evidence for this notion is that in obese T2D patients the presence of NAFLD is associated with more severe atherogenic dyslipidaemia, hyperinsulinaemia and IR in the adipose tissue and the liver compared to NAFLD-free control subjects[93]. A recent study on the molecular effectors of NAFLD-associated IR[94] has shown that fatty liver induce local and systemic chronic inflammation and IR *via* an altered protein secretory profile, notably including excess fetuin B, and that the prevention of fatty liver is a rational target for reducing the development of impaired glucose disposal in over-nourished individuals. Consistently, strategies aimed at reducing the development of fatty liver *via* antisense oligonucleotides against β-catenin may protect mice from diet-induced fatty liver and hepatic and peripheral IR[95].

In the setting of obesity or T2DM/pre-diabetes, the presence of NAFLD often develops in concert with homologous fatty changes of the pancreas[96-101]. Nonalcoholic fatty pancreas (NAFP) may be diagnosed by imaging techniques[102], and it is common in the general population[99]. The role of NAFP as a pathogenic mediator for the association between NAFLD and T2D risk has recently been reviewed[103-105].

CVD in NAFLD: In principle, the increased CVD risk seen in patients with NAFLD may result from a shared pathophysiological background, such as systemic IR and MetS. Such a view, however, would conflict with those studies reporting that NAFLD *per se*, regardless of coexisting MetS features, exposes to excess CVD risk[67]. Consistently, patients with more "*severe*" NAFLD are exposed to an increased risk of fatal and non-fatal CVD events compared to NAFLD-free controls[88]. In agreement, NASH, rather than simple steatosis, is associated with endothelial damage and over-expression of multiple atherogenic mediators and regulators of blood pressure[106]. Collectively, these data would support that NASH, as opposed to simple steatosis, is associated with a higher CVD risk. However, this conclusion needs to be further confirmed by future larger prospective studies.

Consistent with the notion reported above, the higher CVD risk seen in patients with NAFLD may result from increasing fibrosis stage, steatosis grade or oxidative stress[38]. Two studies indirectly confirmed this contention. The first study found that both all-cause and CVD mortality were higher especially in NAFLD patients with coexisting MetS features; conversely, the risk of mortality of metabolically-normal NAFLD patients was similar to that of the cohort without liver disease[77]. The second study found that the MetS-associated NAFLD was associated with a higher risk of CVD, T2D and increased cardiac mass, whereas NAFLD without MetS [*i.e.,* a condition that was more frequently associated with the I148M variant of the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*) polymorphism] was not[107].

Evidence for fibrosis stage playing a role in CVD risk derives from a cross-sectional study conducted in 1874 healthy European adolescents belonging to the general population. In that study, NAFLD individuals had more advanced non-invasively assessed liver fibrosis and worse cardiometabolic risk profiles independent of potential confounders[108].

Increasing liver fat content is also associated with worsening atherogenic dyslipidaemia and dysglycaemia; accordingly, it can reasonably be hypothesized that the quantity of fatty liver may dictate the risk of cardiometabolic outcomes in patients with NAFLD[109]. Consistent with this hypothesis, a recent cross-sectional study reported that, independent of NASH, increased liver fat content was associated with increased rates of MetS features in NAFLD patients[110].

Given that free fatty acids may induce systemic/hepatic IR, oxidative stress and increased synthesis of pro-thrombotic markers in cultured human hepatocytes[111], it can also be speculated that increased oxidative stress may be a critical contributor to CVD risk seen in NAFLD patients. Consistently, NASH was associated with increased oxidative stress and subclinical atherosclerosis in a recent study[112].

Finally, it is conceivable that also the chemical nature of hepatic fat content may play a role in the link between NAFLD and CVD risk. A study reported that three chemical compounds (11,12-dihydroxy-eicosatrienoic acid, 13,14-dihydro-15-keto prostaglandin D2 and 20-carboxy arachidonic acid) are more abundant in NASH than in simple steatosis[113]. Moreover, analysis of the human hepatic lipidome showed that similar increases in liver fat content and NASH were associated with a metabolically harmful saturated, ceramide-enriched liver lipidome in "MetS-related NAFLD" but not in "PNPLA3-related NAFLD", accounting for the finding that the former, rather than the latter, was associated with an increased risk of T2D and CVD[114].

HCV INFECTION

Epidemiology, risk factors and natural history of HCV infection

The worldwide prevalence of HCV infection approximates 1.6%, affecting about 115 million individuals, 80 million of whom are deemed to be viremic[115]. Globally, the G1 genotype is the most common and accounts for roughly one in two of all HCV infections in adults, followed by G3, G2, G4, G6, and G5 genotypes[115].

Traditional risk factors for HCV infection are: assumption of intravenous and intranasal illicit drugs; hemodialysis; cancer; paid blood donations; having

received blood products prior to 1990; high-risk sexual behavior, tattoos or body piercings, working in health care[116]. Presently, in developed countries, the majority of new HCV infections are observed in individuals who inject drugs and in men who have sex with men[116].

Three to four million people are newly infected annually and approximately 350000 people per year die from HCV-related causes. HIV-HCV co-infected individuals sum up to approximately 2500000 worldwide, half of whom belonging to special populations, such as drug injectors[117].

Given that chronicity is the major complication of acute HCV infection, which can lead to many serious liver-related and extra-hepatic outcomes, HCV to date is a significant global public health burden[118].

Over the last few years, the advent of new direct antiviral agents (DAA) has revolutionized the HCV treatment by increasing sustained viral response (SVR) rates from approximately 50% to > 90%. Given that SVR plays a key role in HCV natural history, a multicenter observational study recently found that the survival rates of HCV-related cirrhotic patients who had attained SVR were essentially similar to those in the general population, justifying DAA treatment being administered as earliest as possible to achieve the maximal benefit in HCV-related, compensated cirrhosis[119].

Epidemiological evidence for an association of HCV infection with T2D

HCV is a systemic disease featuring a variety of extra-hepatic manifestations, among which T2D plays a leading role[60,120,121]. Consistently, T2D is the second most prevalent extra-hepatic manifestation, affecting up to 15% of HCV-infected patients[122].

Several cross-sectional studies have shown that patients with HCV-related cirrhosis have a higher prevalence of T2D than those with cirrhosis unrelated to HCV infection[123-125]. Similar results have also been reported in the setting of liver transplantation[126-129], and in patients with all stages of chronic HCV, irrespective of the presence of cirrhosis, suggesting that the connection of HCV infection with IR/T2D was independent of the stage of liver disease[130-136]. A cross-sectional study investigating the relationship between HCV and T2D at the population level showed that T2D occurred more often in HCV-infected persons aged more than 40 years[137]. Consistently, other cross-sectional studies, involving T2D patients, showed that these patients had an increased prevalence of HCV infection[138-140].

Stronger evidence for a possible causal link between HCV and T2D came from longitudinal studies and meta-analyses. A United States prospective case-cohort study showed that pre-existing HCV infection increased the risk of new-onset T2D in persons with coexisting T2D risk factors[141]. A subsequent community-based longitudinal study, involving 4958 non-diabetic Taiwanese individuals, confirmed a significant temporal relationship between prior HCV infection and risk of new-onset T2D[142]. A recent meta-analysis of 31 studies (involving a total of 10388 T2D cases among 61843 HCV individuals and 42358 T2D cases among 202130 HCV-free controls) found that the pooled estimates for odds ratios for new-onset T2D among individuals with HCV infection were 1.58 (95%CI: 1.30-1.86)[122]. These findings are strikingly similar to the results of a previous meta-analysis that showed an approximately 1.7-fold excess risk of new-onset T2D in HCV-infected cases as compared to non-infected controls[143].

Interestingly, a recent study has estimated that the direct medical costs associated with HCV-related T2D were 443 million dollars yearly in the United States, making T2D the most expensive extra-hepatic complication of HCV[122]. Moreover, in patients with HCV, the coexistence of T2D is strongly associated with an increased risk of liver fibrosis progression[132,144-148], cirrhosis and HCC development[149-152], poor liver-related outcomes[152], lower response rates to peginteferon/ribavirin treatment[145,153-155], and may also partly account for the increased CVD risk[156,157].

Given the substantial health and economic burden of T2D among patients with HCV, treatment strategies aimed at eradicating HCV should also consider the potential benefits in terms of T2D prevention/resolution. Of note, studies have shown that HCV clearance with interferon-based treatment regimens may improve IR and pancreatic beta cell function[158,159], reduce T2D development[160], and improve renal and CVD outcomes[161,162]. Although new DAAs allow HCV eradication in the vast majority of HCV patients, little is currently known on the effects of DAAs on glucose metabolism[163]. One study showed that SVR obtained with the first-wave protease inhibitor telaprevir was associated with IR improvement[164]. Moreover, preliminary studies showed that DAAs significantly reduced plasma glucose and hemoglobin A1c (HbA1c) levels in both HCV patients with and without T2D[165,166].

Epidemiological evidence for an association of HCV infection with CVD

HCV has been isolated from the myocardium of patients with myocarditis and dilated cardiomyopathy and from carotid atherosclerotic plaques, suggesting a direct role of HCV in cardiac dysfunction and accelerated atherogenesis[167-170]. Moreover, a higher prevalence of traditional CVD risk factors and MetS features, such as IR/T2D and fatty liver, have been reported in patients with HCV compared to uninfected controls[33,60,122,171]. A growing body of evidence also identifies HCV infection as a potential risk factor for subclinical and clinical CVD complications[156,157].

A proof-of-concept study among 217 Japanese HCV-positive patients without overt CAD demonstrated that myocardial perfusion defects, as detected by scintigraphy, are almost universal in HCV patients, are associated with liver disease activity and HCV-

RNA levels, and improve following viral eradication[172]. Moreover, functional and morphological myocardial changes (as assessed by cardiac magnetic resonance) are common among HCV patients with end-stage liver disease[173]. A population-based cohort study showed that HCV-infected patients have a higher arterial stiffness than HCV-negative control subjects[174]. In addition, multiple cross-sectional studies, which examined carotid atherosclerosis, provided further strong evidence of a relationship between HCV and subclinical atherosclerosis[156,175-179]. Of note, a recent meta-analysis confirmed an association between HCV infection and carotid atherosclerosis[157]. The severity of hepatic steatosis and fibrosis and HCV viral load are among the strong predictors of carotid atherosclerosis[157,178,179].

Importantly, HCV infection is also associated with increased CVD morbidity and mortality. A large study conducted among US Veterans showed that, despite a more favorable cardio-metabolic risk profile, HCV-infected patients had a 1.25-fold higher risk of CAD than uninfected controls[180]. Similarly, a recent Taiwanese community-based study showed that HCV was significantly associated with ischemic electrocardiogram findings[181], and a United States population study found a significant association between HCV infection and the risk of congestive heart failure[182]. Several case-control studies subsequently confirmed that HCV infection predicted CAD and was independently associated with a greater severity of coronary stenoses[183-186], also among HCV-HIV co-infected patients and dialysis patients[187,188].

Similar findings were reported from studies evaluating the risk of cerebro vascular events among HCV patients[189-191]. For instance, a large Taiwanese population-based cohort study showed that HCV-infected patients had a 1.3-fold higher risk of ischemic stroke than HCV-free individuals[189]. Of note, HCV eradication (with interferon-based therapy) has reduced the long-term risk of ischemic stroke and acute coronary syndrome in HCV patients[161,162,191].

A recent meta-analysis of 8 observational studies confirmed that HCV infection was associated with increased CVD morbidity, and the strength of this association was similar for CVD and cerebro vascular events[157].

Worryingly, some studies also suggested a significant association between HCV and cerebral hemorrhages[192,193], and a recent large Taiwanese cohort study extended the association between HCV and atherosclerosis by showing that HCV-infected patients had an increased risk of peripheral artery disease compared to non-HCV control subjects[194].

Finally, a number of studies reported that HCV infection was independently associated with an increased CVD mortality[157,195-197] also in HIV co-infected patients and in dialysis patients[198,199].

Pathogenic mechanisms of T2D and CVD in HCV infection

A significant proportion of HCV-related morbidity and mortality may result from the coexistence of T2D and CVD in the development of which HCV may play a pathogenic role; moreover, HCV may also induce fatty liver, which in its turn, further increases CVD risk[200], especially in those with T2D and hypertension[157].

The main reason why HCV is a "successful pathogen" is that it exploits its host's metabolism to build up viral particles. In so doing, HCV infection has developed a set of metabolic abnormalities collectively referred to as "hepatitis C-associated dysmetabolic syndrome" (HCADS)[171]. HCADS includes fatty liver, hyperuricaemia, hypocholesterolaemia, IR, hypertension and visceral overweight/obesity. Such metabolic disorders may best be interpreted as a "Darwinian strategy", favoring HCV survival at the expense of the host's metabolism. The finding of expanded visceral adipose tissue in HCV-infected patients is consistent with the hepatic and extra-hepatic origins of IR discussed above, and prompts further research regarding the potential ability of HCV to infect the adipose tissue[60].

Similar to the pathogenic model of T2D developing in the setting of NAFLD, HCV may exacerbate IR eventually leading to dysfunction/damage of pancreatic beta cells and development of overt T2D in most patients over time[121]. Conversely, HCV eradication may result in IR improvement, although definite evidence for this conclusion is eagerly awaited[201].

HCV antigens, notably including the core protein, play a key role in determining IR by interfering with the AKT signaling pathway, through the action of both pro-inflammatory cytokines [*e.g.,* tumor necrosis factor-alfa and interleukin-6 (IL-6)] and suppressors of cytokine signaling[202]. The site of IR is not only hepatic but also extra-hepatic, mainly in the skeletal muscles, correlates with subcutaneous, rather than visceral adiposity, and is independent of liver fat content[60,171].

The role of specific viral genotypes in the development of IR is also increasingly appreciated. An extensive French study reported that IR was significantly associated with G1 and G4 genotypes, high HCV-RNA viral load and significant hepatic fibrosis, irrespective of fatty liver[134].

T2D and IR are strong predictors of faster progression of liver fibrosis and impaired response rates to HCV treatment[153,203]. Patients with HCV-related cirrhosis and T2D have an increased susceptibility to developing hepatic encephalopathy and HCC; moreover, concurrent T2D accounts for a persistently higher HCC risk even in those patients attaining SVR[204-206]. Theoretically, in patients with chronic HCV infection, a better glycaemic control could improve the prognosis and the response rate to HCV treatment, although direct evidence for this is limited.

Beneficial effects of antiviral treatment on IR and T2D are increasingly identified. By evaluating paired

pre-treatment and post-treatment HOMA-estimated IR measurements in 1038 non-diabetic HCV patients, a study found that SVR was associated with a significant IR improvement in HCV patients infected with G1 genotype (but not in those infected with G2/3); IR improvement was independent of body weight, serum transaminase and lipid level changes, suggesting that G1 genotype might directly promote IR, independent of host metabolic factors, and might be improved following HCV eradication[207].

A preliminary clinical trial of 29 patients with T2D who were receiving different interferon-free regimens reported that fasting glucose and HbA1c levels were significantly reduced by the treatment, irrespective of the DAA used, HCV genotype, body weight and HIV status. Consistently, the dosages of hypoglycemic drugs were reduced in about a quarter of these patients[165].

T2D is not the only metabolic disease observed in the setting of HCV infection. Over time, several metabolic features of what is now alluded to as the HCADS have been increasingly identified. The HCV-related fatty liver, which is one of such features, was first identified as a distinct disease entity. Data comparing fatty liver both in the viral setting and NAFLD suggest that IR is a prominent feature specifically associated with HCV infection[7].

All HCV genotypes share multiple "NAFLD-like" steatogenic mechanisms, such as increased availability of lipogenic substrates and *de novo* lipogenesis; decreased oxidation of fatty substrates and export of fatty substrates. However, G3 genotype induces more prominent derangements in molecular mediators of steatogenesis (*e.g.,* microsomal triglyceride transfer protein; peroxisome proliferator-activated receptor alpha; sterol regulatory element-binding proteins and phosphatase and tensin homologue), thus amplifying those steatogenic mechanisms, which also take place in NAFLD. As a result of this, fatty liver is more frequently associated with G3 genotype and is, therefore, designated as "viral fatty liver" as opposed to fatty liver occurring in HCV non-3 genotypes and particularly G1 genotype, which is more closely associated with host's features[171,203,208-210].

Among the various features of HCADS, HCV-related fatty liver has a distinct, prominent clinical impact in as much as it accelerates hepatic fibrogenesis; impairs the response rates to interferon-alpha and ribavirin treatments; increases the risk of developing HCC and predisposes to accelerated atherogenesis[33,156,171,179].

HIV INFECTION

Epidemiology, risk factors and natural history of HIV infection

Recent UNAIDS estimates indicate than in 2015 there were approximately 2.1 million new cases of HIV infections worldwide, adding up to a total of approximately 37 million people already living with HIV[211]. Against these dramatic features, over the last 15 years, HIV medicine gained impressively positive results in terms of improved life expectancy for HIV-infected adults in Western countries. This finding results from major changes in the natural history of HIV disease and in the efficacy of anti-retroviral therapy (ART)[212,213]. Scale-up of the ART is on a fast-track trajectory that has surpassed expectations. Global coverage of ART reached approximately 46% at the end of 2015 (with the greatest gains in the world's most affected region, Eastern and Southern Africa)[214]. Several recent studies have suggested that the life expectancy of HIV-infected patients may approach that of the general population, particularly among patients who initiated ART at earlier disease stages[215]. Simultaneously, HIV seroconversion among older age persons is increasingly recognized, partly due to a lower perception of sexual risk in older people[216]. As a result of these changes, what we observe is that HIV-infected persons are more likely to be older and have an increased burden of age-related comorbidities compared to persons of a similar age who have been infected by HIV more recently[217].

While this "graying" of the HIV-infected population is to be welcomed as an indication of improved treatment and survival rates, the increasing number of older HIV-infected patients with increased rates of comorbidities presents new challenges for patient care. Cardio metabolic complications, such as CVD, atherogenic dyslipidemia and glucose intolerance/T2D, are very common in older HIV-infected patients, and justify the onset of multidisciplinary metabolic clinics for the management of patients with chronic HIV-related co-morbidities[218].

Epidemiological evidence for an association of HIV infection with T2D

Patients with HIV are at higher risk of new-onset T2D compared to the general adult population[219]. The reasons for this increased T2D risk are not entirely understood, but may include the use of some ARTs[220], the presence of HCV co-infection[221], and the HIV-induced systemic chronic inflammation[222,223]. Untreated HIV is associated with higher levels of multiple pro-inflammatory biomarkers, and initiation of ART results in a decline, but does not normalize plasma pro-inflammatory biomarkers[224]. The occurrence of IR, which is causally associated with the development of T2D, was one of the first metabolic disorders associated with HIV disease and treatments[225].

T2D development can be, at least in part, linked to an underlying chronic inflammation. Population-based studies suggested that higher levels of IL-6 and C-reactive protein (CRP) are independently associated with an increased risk of new-onset T2D[226]. Cross-sectional studies also reported a significant and positive association between plasma CRP levels and IR in

non-diabetic individuals[227]. Moreover, it has been shown that in healthy volunteers the administration of subcutaneous recombinant human IL-6 induced a dose-dependent increase in fasting glucose levels[228]. A recent retrospective cohort study involving 3695 non-diabetic participants, who took ART and were followed-up for an average of 4.6 years, found that higher circulating CRP and IL-6 levels were significantly associated with an increased risk of new-onset T2D. Other common risk factors for new-onset T2D in this cohort of HIV-infected patients were older age, higher body weight, co-infection with hepatitis B or C, non-smoking status and use of lipid-lowering drugs. All these findings suggest that systemic chronic inflammation may contribute to the development of T2D in patients with HIV[229].

Epidemiological evidence for an association of HIV infection with CVD

The burden of CVD is increased in HIV-infected patients[230]. The investigators of the Veterans Aging Cohort Study (VACS) recently analyzed the data collected in 82459 Veterans who were followed-up for a mean period of 5.9 years. When these VACS participants were stratified by the presence or absence of traditional CVD risk factors, HIV-infected patients without risk factors had a two-fold greater risk of incident acute myocardial infarction compared to uninfected control individuals and their risk steeply increased with each additional CVD risk factor added[231].

Although CVD has become a major cause of mortality among HIV-infected patients, outnumbering those figures expected based on the high prevalence of CVD risk factors observed in this patient population, controversy still exists regarding the underlying pathogenic mechanisms of accelerated atherosclerosis in HIV.

Such an increased CVD risk may be likely due to both a direct role of HIV and the dysregulated immunological responses caused by chronic HIV infection[231-233]. Tight control of HIV replication and maintenance of a good CD4 count seem to protect from the risk of incident CVD events. However, there is conflicting evidence regarding the association of the CD4 cell count and the HIV-RNA levels with the risk of CVD[234,235]. The trans-activator of transcription (Tat), *i.e.,* a regulatory protein that enhances the efficiency of viral transcription, may represent a pathogenic mechanism by which HIV may directly induce accelerated atherogenesis[236]. Tat induces the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin[237], which are associated with an increased incidence of CVD in healthy individuals[238]. The levels of these circulating endothelial adhesion molecules are markedly increased in HIV-infected patients, and there is a significant relationship of elevated soluble ICAM-1 levels with HIV disease progression and reduced CD4 cell count[239]. Similarly to Gp120, Tat may also decrease endothelium-dependent vasodilation and nitric oxide synthase secretion in porcine coronary arteries[240].

In addition to the above-mentioned humoral effects, cellular immune activation may also play a role in CVD development among HIV-infected patients[241]. Monocytes are readily infected by HIV and may adhere to the endothelial surface and, eventually, penetrate in the sub-endothelial space and intima[242]. Furthermore, monocytes expressing $CD14^+$ and $CD16^+$ are also prone to a greater pro-inflammatory activity once infected by HIV[243].

Uncertainty still remains as to the extent to which traditional CVD risk factors, immune risk factors, and non-traditional behavioral risk factors contribute to CVD development in HIV-infected individuals. Importantly, the circulating monocyte activation marker sCD163 is associated with high-risk morphology coronary atherosclerotic plaques and arterial inflammation[244,245]. Finally, in specific populations of HIV patients, non-traditional behavioral risk factors, such as cocaine use, may also contribute to accelerated atherogenesis *via* multiple mechanisms[246].

Pathogenic mechanisms of T2D and CVD in HIV infection

In HIV-infected patients the etiology of CVD and T2D reflects the complex and intertwined interactions of multiple factors present in this particularly high-risk patient population, such as the higher prevalence of traditional and non-traditional CVD risk factors, the effects of HIV infection, and the effects of ARTs. In this context, it is reasonable to assume that fatty liver itself might also play a pathogenic role in the development of CVD in patients with HIV.

The adverse effect of the HIV infection on the re-distribution of adipose tissue is known as "lipo-hypertrophy", which mainly consists of the expansion of ectopic fat depots such as abdomen, liver, dorso-cervical region (sometimes with buffalo hump), trunk and heart, which may all contribute to the development of T2D and other MetS features[247].

In patients in whom fatty liver occurs in the context of HIV and/or HCV (*i.e.*, VAFLD), the development of fatty liver may result from a complex interplay between host and viral factors, such as sex, lifestyle habits, IR, MetS, HCV genotype, viral load and ART use[210,248]. In HIV-infected patients, the potential steatogenic effect of ARTs remains controversial[249,250]. Although VAFLD is highly prevalent in HCV/HIV-coinfected patients[251], the natural history of fatty liver observed in these patients remains incompletely understood. A recent study reported that fatty liver was a precursor lesion to hepatic fibrosis[252], suggesting that the effective management of fatty liver might also result in the reduction of hepatic fibrosis progression in patients

with HCV/HIV co-infection[253]. Moreover, IR is a predictor of reduced SVR in both HCV-monoinfected and HCV/HIV-coinfected patients[155]. All these findings further underline the importance of improving our understanding of the independent contribution of fatty liver to the pathophysiology of CVD and T2D in this patient population.

To date, there are very limited data regarding the clinical, biochemical and liver histological features of HIV-related VAFLD; moreover, it remains uncertain whether VAFLD differs in terms of clinical presentation and histological severity from primary NAFLD. A small cross-sectional study showed that, compared to age- and sex-matched patients with primary NAFLD, those with HIV-related VAFLD not only had a more severe metabolic profile but also more severe forms of liver disease[254].

Presently, large prospective studies on VAFLD in HIV are lacking. A very small series of 10 HIV mono-infected VAFLD patients, who were treated either with maraviroc (MRV) plus atazanavir (n = 7) or with vitamin E (n = 3), found that at baseline the histologic degree of hepatic steatosis was 20% and decreased to approximately 10% after treatment. The extent of steatosis improved in all patients, except for two patients who gained body weight. A reduction in hepatic fat content assessed histologically and with magnetic resonance at 48 wk was found both in the atazanavir/MRV and, to a lesser extent, in the Vitamin E treatment groups. The results of this preliminary study suggest a possible role of MRV in the treatment of fatty liver in HIV-infected patients (Guaraldi G *et al*, unpublished data). However, future large randomized clinical trials are needed to confirm these findings.

As lipodystrophy may predispose HIV-infected patients to develop T2D and other MetS features, new prevention and management strategies should be evaluated. For example, avoidance of some ART regimens, containing stavudine and zidovudine, might help in preventing the development of lipoatrophy, but no strategies have proven effective against lipohypertrophy[255]. For patients with established lipoatrophy, switching from ART regimens, containing either stavudine or zidovudine, to an alternative ART regimen could be an empirical choice. Tesamorelin, a synthetic growth-hormone-releasing analogue, has been approved for the treatment of lipohypertrophy. This drug may reduce both abdominal visceral adiposity[256] and liver fat content[257], but its long-term effects on the risk of T2D and CVD remain uncertain.

CONCLUSION

In this narrative review, we have tested the hypothesis that fatty liver represents a shared pathogenic mechanism for the development of CVD and T2D not only in patients with NAFLD but also in those with chronic HCV or HIV infections. To do so, we have followed an innovative multidisciplinary comparative approach, by extending the widely accepted comparison of NAFLD with chronic HCV infection[60,258] to HIV infection.

What we have found is that, the published data are biologically consistent in identifying NAFLD as a major player in the development of T2D and CVD (as schematically shown in Figure 1). Indeed, the clinical burden of NAFLD is not only restricted to liver-related morbidity or mortality, but there is now growing evidence that NAFLD is a multisystem disease, affecting multiple extra-hepatic organs and regulatory, inflammatory and metabolic pathways[259]. Strong evidence indicates that CVD complications frequently dictate the clinical outcomes of NAFLD patients and, therefore, screening for CVD is mandatory in all patients with NAFLD, at least by utilizing a detailed risk factor assessment[15,259-261]. Furthermore, convincing evidence also supports a link between NAFLD and the risk of new-onset T2D[60,259]. Collectively, accumulating evidence suggests that NAFLD is not a simple marker of CVD and T2D, but also plays a part in the pathophysiology of these cardiometabolic complications[38,60,259,261]. NAFLD, especially in its necro-inflammatory variant (NASH), exacerbates systemic/hepatic IR, predisposes to atherogenic dyslipidaemia, and releases several pro-inflammatory, pro-coagulant, pro-oxidant and pro-fibrogenic mediators that play important roles in the development of CVD and T2D[38,60,259,261].

Likewise, HCV and HIV are two globally prevalent pathogens and leading causes of mortality and morbidity worldwide. Patients with chronic HCV infection or HIV infection are at higher risk of developing T2D and CVD[60,157,262,263]. Interestingly, fatty liver is often observed in patients with chronic HCV or HIV infections[60,264] and a wide array of similarities with NAFLD further supports a link of HCV or HIV infections with the risk of T2D and CVD.

On the grounds of data examined here, it is therefore reasonable to assume that fatty liver might be a shared pathological condition which plays a key role in the development of T2D and CVD not only in patients with NAFLD but also in those with chronic HCV or HIV infections. However, further research is required to further corroborate the prognostic role of fatty liver *per se* in the development of T2D and CVD in patients with chronic HCV or HIV infections, and to better elucidate the differences and similarities between NAFLD and VAFLD. Moreover, it remains uncertain whether specific algorithms for CVD/T2D risk assessment[37] should be implemented and validated both in patients with NAFLD and in those with VAFLD.

In the meantime, the findings of this review may disclose new avenues in the clinical and research arenas. Our findings also pave the way for planning future prospective and intervention studies of clinical relevance because, for example, with the success of ARTs, HIV-related co-morbidities, like T2D and CVD,

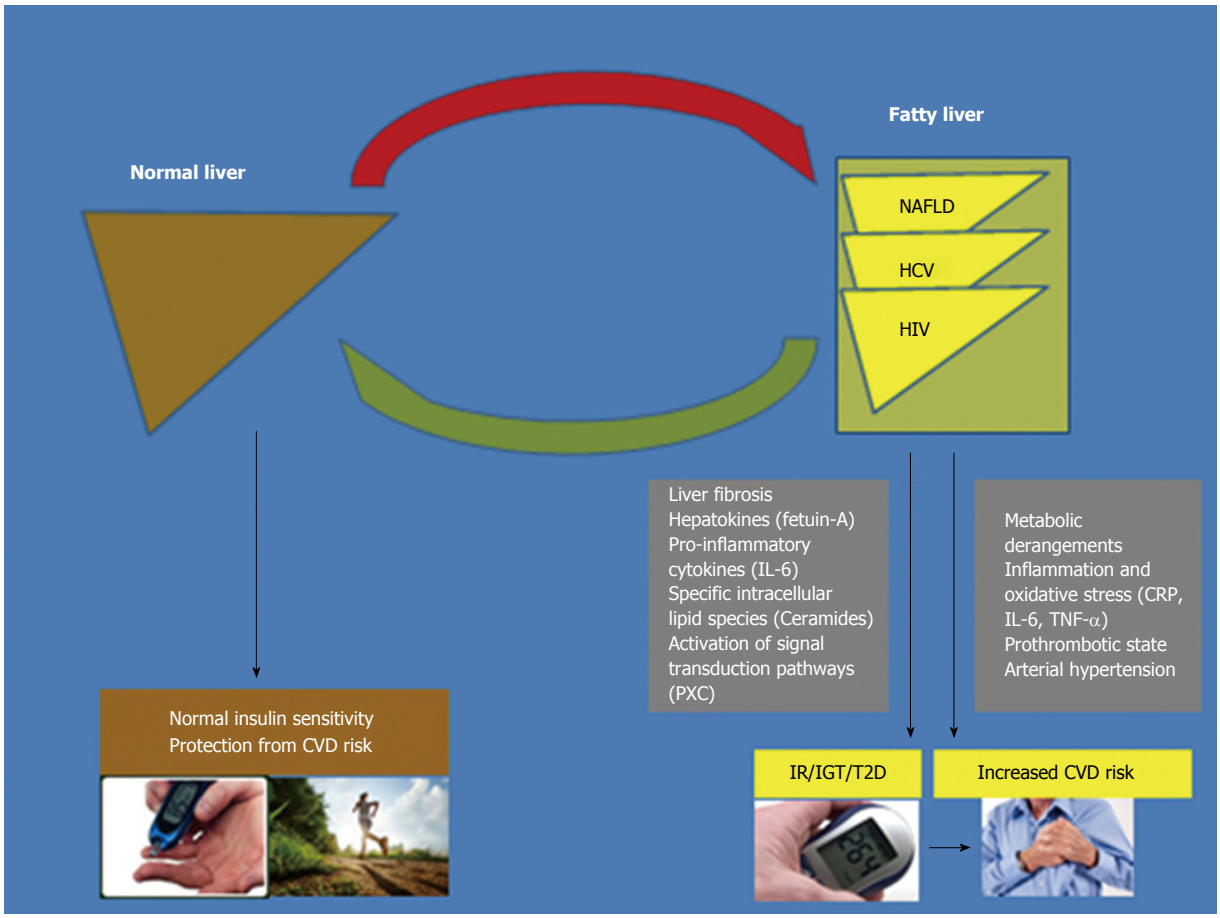

Figure 1 Mechanisms underlying type 2 diabetes and cardiovascular disease in nonalcoholic fatty liver disease and virus-associated fatty liver disease. This cartoon schematically depicts the nonalcoholic fatty liver disease (NAFLD)-derived paradigm supporting a link between NAFLD and the development of type 2 diabetes (T2D) and cardiovascular disease (CVD). Based on this model, the presence of NAFLD per se plays a role in the pathogenesis of these two cardiometabolic complications. This possibly occurs through the systemic release of several pro-inflammatory/pro-coagulant mediators from the steatotic/inflamed/fibrotic liver or through the contribution of NAFLD itself to insulin resistance and atherogenic dyslipidaemia. Virus-associated fatty liver disease (VAFLD) identifies fatty liver associated with either chronic HCV or HIV infections. The novel comparative data presented in this review support the assertion that VAFLD conceptually mimics NAFLD and thus might partly contribute (along with specific viral factors related to HCV and HIV) to the pathogenesis of T2D and CVD also in this group of patients. The putative pathogenic mechanisms illustrated in the right hand side of the figure have been predominantly shown in NAFLD[37,104] and, by inference, should also be investigated in VAFLD.

are of increasing concern[263].

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REVIEW

Epidemiology of pancreatic cancer

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Abstract

Cancer of the pancreas remains one of the deadliest cancer types. Based on the GLOBOCAN 2012 estimates, pancreatic cancer causes more than 331000 deaths per year, ranking as the seventh leading cause of cancer death in both sexes together. Globally, about 338000 people had pancreatic cancer in 2012, making it the 11th most common cancer. The highest incidence and mortality rates of pancreatic cancer are found in developed countries. Trends for pancreatic cancer incidence and mortality varied considerably in the world. A known cause of pancreatic cancer is tobacco smoking. This risk factor is likely to explain some of the international variations and gender differences. The overall five-year survival rate is about 6% (ranges from 2% to 9%), but this vary very small between developed and developing countries. To date, the causes of pancreatic cancer are still insufficiently known, although certain risk factors have been identified, such as smoking, obesity, genetics, diabetes, diet, inactivity. There are no current screening recommendations for pancreatic cancer, so primary prevention is of utmost importance. A better understanding of the etiology and identifying the risk factors is essential for the primary prevention of this disease.

Key words: Pancreatic cancer; Epidemiology; Incidence; Mortality; Trend; Risk factors

Core tip: Pancreatic cancer is the one of leading causes of cancer mortality and one of the most lethal malignant neoplasms across the world. The highest incidence and mortality rates of pancreatic cancer are found in developed countries. The estimated 5-year survival rate for pancreatic cancer is about 5%. The causes of pancreatic cancer are still insufficiently known, although certain risk factors have been identified, such as cigarette smoking, positive family history and genetics, diabetes mellitus, obesity, dietary factors, alcohol use, physical inactivity. There are no current screening recommendations for pancreatic cancer, so primary prevention is of utmost importance.

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INTRODUCTION

Pancreatic cancer is the one of leading causes of cancer mortality in developed countries and one of the most lethal malignant neoplasms across the world[1-3]. The two main tumor types of pancreatic cancer are adenocarcinoma (that accounts for about 85% of cases), and pancreatic endocrine tumors (which are make up for less than 5% of all cases)[1,4,5].

Based on the GLOBOCAN 2012 estimates, pancreatic cancer causes more than 331000 deaths per year (accounts for 4.0% of all deaths), ranking as the seventh leading cause of cancer death in both sexes together[1]. About 338000 people had pancreatic cancer in 2012, making it the 11th most common cancer[1,2]. The estimated 5-year survival rate for pancreatic cancer is less than 5%[2,4]. The incidence and mortality of pancreatic cancer worldwide correlated with increasing age and was slightly more common in men than in women[1-3]. In the past decades, pancreatic cancer mortality has been increasing in both genders (for example, in the United States, European countries, Japan, China)[1,3,6-8].

The causes of pancreatic cancer are still insufficiently known, although certain risk factors have been identified, such as cigarette smoking, positive family history and genetics, diabetes mellitus, obesity, dietary factors, alcohol use, physical inactivity[9-12].

Understanding the epidemiology of pancreatic cancer could be the key to elucidating the etiology of pancreatic cancer and thus the cornerstone of developing a prevention strategy.

INCIDENCE

The incidence of pancreatic cancer varies greatly across regions and populations. Incidence rates for pancreatic cancer in 2012 were highest in Northern America (7.4 per 100000 people) and Western Europe (7.3 per 100000 people), followed by other regions in Europe and Australia/New Zealand (equally about 6.5 per 100000 people) (Figure 1)[1]. The lowest rates (about 1.0 per 100000 people) were observed in Middle Africa and South-Central Asia. Differences in incidence rates were twenty fold between the populations with the highest rate (Czech Republic - 9.7), and the population with the lowest rate (Pakistan - 0.5). More than half of new cases (55.5%) were registered in more developed regions[1]. Slightly less than half (41.0%; 139363 of cases) of all new cases of pancreatic cancer in 2012 were recorded in the countries of Asia.

There are significant geographic variations in the incidence of pancreatic cancer by genders[1,2,6-8]. The incidence rate of pancreatic cancer among men in 2012 was 4.9 per 100000, and among women 3.6 per 100000 (Figure 2)[1]. In men, the risk of developing pancreatic cancer was high in Armenia (11.9) and Czech Republic (11.8), Slovakia and Hungary (equally - 11.5), then in Japan and Lithuania (equally - 10.6) (Figure 2A). In contrast, the risk of contracting pancreatic cancer in men was the lowest in Pakistan (0.5) and Guinea (0.4). The regions with the highest incidence rates of pancreatic cancer in women were Northern America (6.4 per 100000), Western Europe (6.3), and Northern Europe and Australia/New Zealand (5.9 and 5.4, respectively) (Figure 1)[1]. The lowest rates of pancreatic cancer incidence (less than 1.0 per 100000) were in Middle Africa and Polynesia. Women living in Hungary, Denmark, Finland and Armenia have the greatest risk (aproximatelly 7.5 per 100000) of dying from pancreatic cancer, while women in Tanzania, Guinea, Cameroon, Namibia and Pakistan have the lowest disease risk (less than 1.0 per 100000) (Figure 2B).

The incidence rates for both sexes increase with age, the highest in older than 70 years[3,4,6]. It is predominantly a disease of the elderly, and almost 90% of all cases are diagnosed after the age of 55 years[6,13].

Although it is not possible to fully explain the differences in the incidence of pancreatic cancer in different parts of the world, most of the international variation in the incidence of pancreatic cancer has been attributed to exposure to known or suspected risk factors related to lifestyle or the environment[9,10,13]. Tobacco smoking is likely to explain these international variations and gender differences[11]. Some findings may be indicating that obesity may have some effect on differences[14]. Additionally, some findings indicate the role of aging and hereditary and genetic factors. The reasons for higher incidence of pancreatic cancer in men are still insufficiently known: women are either less prone to these kinds of malignant tumors, or are less exposed to risk factors from the environment responsible for their occurrence[9,15]. Besides that, international differences probably reflect diagnostic capacity and the change in use of various diagnostic modalities[16]. In 2012, Europe carried one third of the overall incidence, which likely reflected the more accurate diagnosis of pancreatic cancer rather than etiology[17]. It should be noted that some differences in incidence of pancreatic cancer around the world may be attributed to the quality of registries, which coverage, completeness and accuracy varies by country[18].

MORTALITY

International mortality rates for pancreatic cancer vary significantly in different areas. Rates of mortality from pancreatic cancer in 2012 in both genders were highest in Northern America (6.9 per 100000 people) and Western Europe (6.8), followed by other European regions and Australia/New Zealand (approximately - 6.0, respectively) (Figure 1)[1]. The lowest mortality was

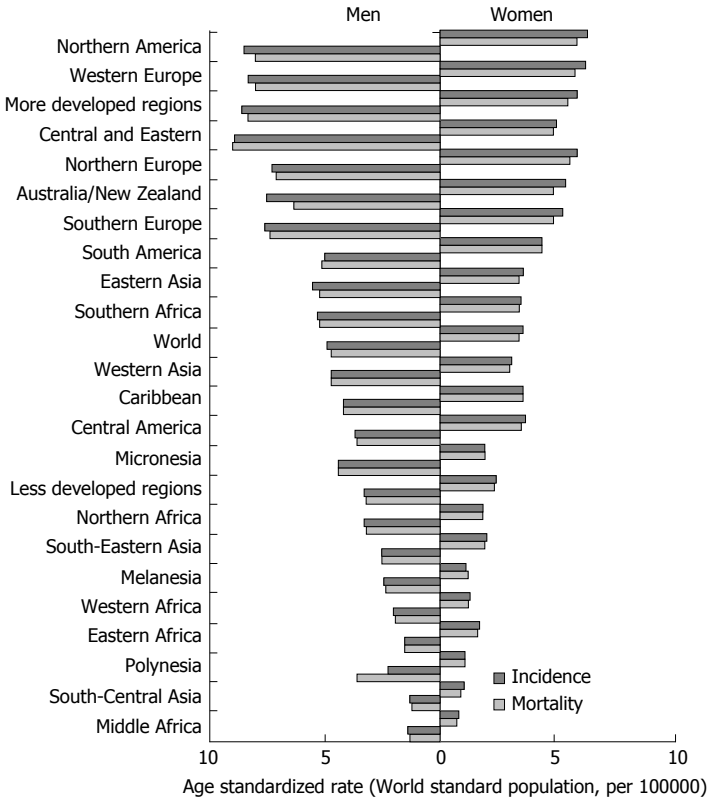

Figure 1 Pancreatic cancer incidence and mortality in men and women, by regions, GLOBOCAN 2012 estimates.

recorded in the countries in Middle Africa and South-Central Asia (less than 1.0 per 1000000 people). The differences in mortality rates were nearly fifty fold between the populations with the highest and lowest rate (Armenia *vs* Tanzania: 8.9 *vs* 0.2). More than one third (111029 deaths) of all deceased from pancreatic cancer are residents of Europe. Slightly less than half (41.5%; 137251 deaths) of all deaths from pancreatic cancer were recorded in 2012 in Asian countries[1]. More than half (55.8%, 184429 deaths) of deceased of pancreatic cancer were registered in more developed regions. At least deaths were registered in Micronesia/Polynesia. The least number of deaths was registered in Micronesia/Polynesia.

Mortality of pancreatic cancer in both genders increases with age, and almost 90% of all deaths are registered after the age of 55 years[1,3]. The highest mortality rates in 2012 in males were recorded in Central and Eastern Europe (Latvia - 11.9, Hungary - 11.5) (Figure 3A)[1]. The mortality from pancreatic cancer was lowest (less than 1.0 per 100000 people) in Belize and Bahrain. The highest mortality rates in 2012 in females were recorded in Hungary (7.5) and Malta (7.2) (Figure 3B)[1]. The mortality from pancreatic cancer was lowest in women in Belize (0.8).

Mortality of pancreatic cancer is almost identical with its incidence, because it is one of the most fatal malignant tumors[19,20]. Reasons for the substantial differences in mortality rates of pancreatic cancer were not completely elucidated. Differences in rates of incidence can be apparent and specious. Specious differences may arise as a result of changes in the diagnosis of diseases and causes of death, as a result of a real shift in the incidence and/or fatality. Data on the incidence/mortality published by WHO are not of the same quality in all countries[18]. Although the quality (accuracy and completeness of cause of death registration, primarily) and the coverage of information in most developing countries can be considered limited, the registry often remains the only available source. Symptoms, signs and insufficiently defined conditions as the underlying cause of death are significantly more often mentioned in Serbia, the Russian Federation and Greece than in more developed countries such as the United states of America, United Kingdom, and Finland, which points to the need for a cautious interpretation of the data statistics of mortality in international comparisons[18]. Pancreatic cancer is difficult to diagnose. Malignant pancreatic neoplasm was among the most common cancers detected at autopsy studies[16,21]. It is known that for pancreatic cancer there is no workable modality of screening, early detection and effective treatment, which has the consequence of survival rates varying very little between developed and developing countries[22]. Current available treatment options for pancreatic cancer are limited. Due to the advanced stage at diagnosis, 80%-90% of patients have unresectable tumours and long-term survival after surgical resection is poor[13,19,23].

High smoking prevalence has been widely recognized as the main contributor to the high mortality rates of

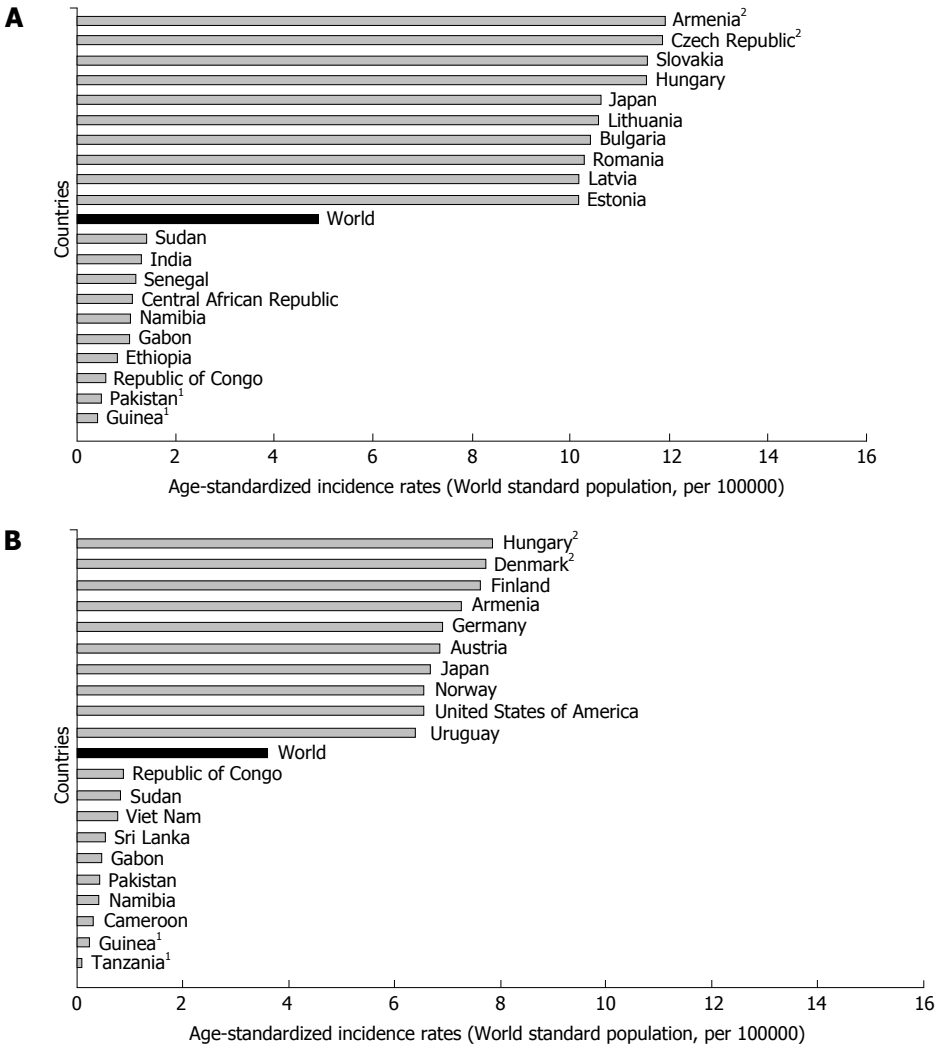

Figure 2 Pancreatic cancer incidence in men (A) and women (B), GLOBOCAN 2012 estimates. [1]Country with the lowest mortality rates; [2]Country with the highest mortality rates. GLOBOCAN 2012 estimates[1].

pancreatic cancer[11,24]. Numerous evidence support that diet (animal fat and meat consumption, *etc.*) plays a role in the development of pancreatic cancer[25,26]. In addition, the highest rates for pancreatic cancer mortality in eastern European countries suggested that other factors (including prevalence of diabetes, obesity, alcohol use) could influence the mortality of pancreatic cancer[27].

TEMPORAL TRENDS

Trends for pancreatic cancer mortality varied considerably in the world. Figure 4 represent pancreatic cancer mortality trends in 8 selected countries using official data for pancreatic cancer abstracted from the WHO database over the period 1955 to 2012[1]. For men in most developed countries (United States, the United Kingdom, Australia, Japan and Finland), a rise was observed between the early 1955 and the late 1980s, followed by a leveling off since 1990s (Figure 4A). Thus, rates for men in Eastern and Southern Europe (Hungary and Greece) rose continuously over the observed period. For women in the United Kingdom, Finland, Japan, United States and Australia, rates tended to rise up to the early 1990s, and to level off thereafter (Figure 4B). In Eastern and Southern European countries (such as Hungary and Greece), rates rose from the early 1970s to onwards.

In the United States, whites and blacks experienced opposite trends in pancreatic cancer death rates between 1975 and 2013[28]. In whites, pancreatic cancer death rates decreased by 0.2% per year from 1095 to 1996 and then increased by 0.4% per year through 2013. In contrast, the rates among blacks increased between 1975 and the late 1990s and then decreased thereafter. Also, in last decades a decreasing trend was found in both sexes in the United Kingdom, Sweden, Australia, the Netherlands, Mexico[13]. An increasing trend in both sexes was noticed in France, Japan, Brazil, Republic of Korea, and most countries of Central (Germany, Hungary), Eastern (Russian Federation, Poland, Romania, Serbia), and Southern Europe (Italy, Spain)[13,29-31].

To date, however, temporal trends in pancreatic

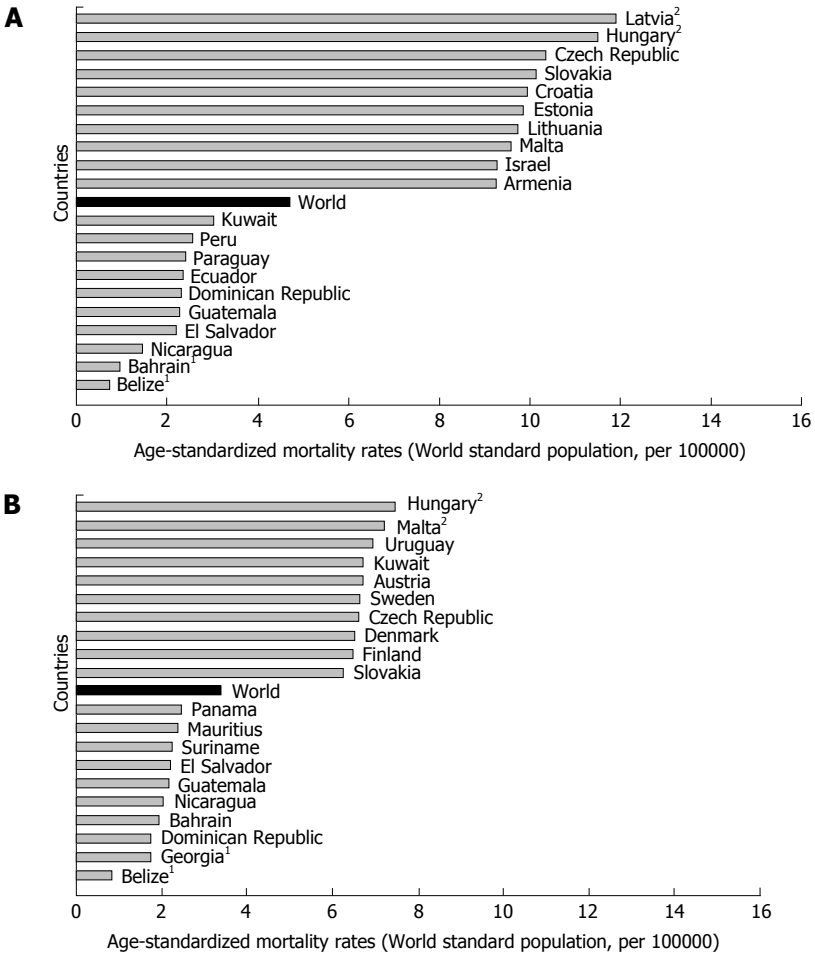

Figure 3 Pancreatic cancer mortality in men (A) and women (B), GLOBOCAN 2012 estimates. [1]Country with the lowest mortality rates; [2]Country with the highest mortality rates. GLOBOCAN 2012 estimates[1].

cancer incidence and mortality have not been well understood. In both sexes, temporal trends in cigarette smoking prevalence were related to temporal trends in pancreatic cancer mortality with a roughly lag of several decades[9,11,24]. Decreased smoking, particularly in men, has been widely recognized as the main contributor to the decreases in mortality trends from pancreatic cancer in developed countries[24,32]. The mortality rate of pancreatic cancer began to decline earlier in the countries where the tobacco control started to be implemented earlier (such as United States, the United Kingdom, Australia). However, the recent increasing trends in some countries in the European Union suggest that other factors (including mainly obesity, physical inactivity, diabetes, and dietary factors), besides smoking, may have influenced the pancreatic cancer mortality[20,22,31,33]. In recent years, people in developing countries adopt lifestyles and behaviors that are typical for developed countries, such as cigarette smoking, higher consumption of saturated fat and calorie-rich foods, and reduced physical activity[25,27,34]. Besides, the trends might be due to disparities in socioeconomic circumstances between high- and low-income areas. Improved diagnostic and death certification of the disease might also partially explain the observed figures, at least part of the recent trends in some countries of Southern, Central and Eastern Europe[18,27,31]. It is unlikely, however, that progress in the treatment of pancreatic cancer could have a significant impact on mortality trends, also given the negligible changes in survival over the last decades[19,22,23,35]. Also, it is known that the changes in coding of pancreatic cancer had minimal influence on mortality trends of pancreatic cancer in the second half of the XX century[18,36].

SURVIVAL

Cancer of the pancreas remains one of the most deadly common cancer types: the Mortality/Incidence ratio is 98%[1]. The overall five-year survival rate is about 6% (ranges from 2% to 9%), but this partly reflects varying data quality worldwide[37,38]. For pancreatic cancer, survival rates vary very small between developed and developing countries[37].

Based on the United States National Cancer Institute' data for pancreatic cancer in both sexes and all races, 9.4% are diagnosed at the local stage while the 5-year survival for localized disease was 29.3%

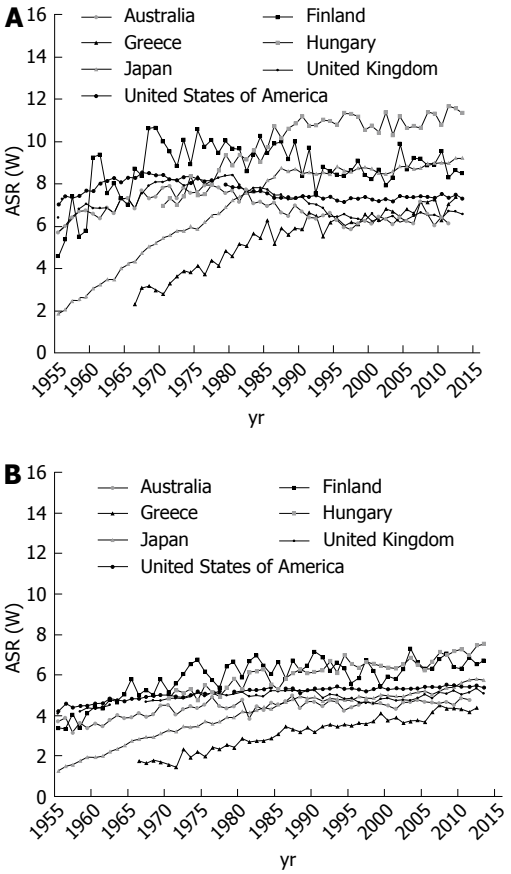

Figure 4 Pancreatic cancer mortality trends among men (A) and women (B) in selected countries. GLOBOCAN 2012 estimates[1].

during 2006-2012[3]. More than half (52%) of all cases were diagnosed at the distant stage with the 5-year survival rates of 2.6%.

Some intercountry survival differences for pancreatic cancers exist over Europe: 5-year survival rate was less than 3% in both sexes in England and Wales[39], Denmark and Sweden - 3.8%[22], in Italy - 1.2%[40]. EUROCARE Working Group analyzed the survival of cancer patients diagnosed from 1990 to 1994 in 22 European countries and showed that 5-year survival rates were highest in men in Estonia (7.0%) and in women in Czech Republic (7.5%), while lowest survival rates were recorded in men in Malta (0.0%) and in women in Slovenia (1.3%)[37]. Survival from pancreatic cancer in Germany in the early 21st century was 9.0%[35].

Survival rates of pancreatic cancer in population are affected by many factors, such as the type of cancer, the staging at the time of diagnosis, serum albumin level, and tumor size, treatment modality, availability and differences in health care systems, and other factors including age, sex, overall health, lifestyle[23,37,41-50]. Besides, pancreatic cancer survival rates could be influenced by factors such as the validity of cancer registry, exhaustiveness and quality of registration data, completeness of follow-up[22,51].

ETIOLOGY AND RISK FACTORS

To date, the causes of pancreatic cancer are still insufficiently known, although certain risk factors have been identified. People are at higher risk to develop pancreatic cancer with any of the risk factors such as smoking, obesity, genetics, diabetes, diet, inactivity[9,12,13,52].

In the study in the United Kingdom in 2011 it was estimated that around 26.2% of pancreatic cancers in men and 31.0% in women was linked to tobacco smoking[9]. The International Agency for Research on Cancer confirmed that smoking is causally associated with pancreatic cancer[11,33]. A recent meta-analysis that included 82 studies found that the risk of pancreatic cancer was RR = 1.7 for current and RR = 1.2 for former smokers[53,54]. Cigarette smoking causes an increase in the risk of pancreatic cancer by 75 percent compared to non-smokers, and the risk persists for at least 10 years after smoking cessation[34,54]. The European (EPIC) study showed in 2012 that risk increased for every five cigarettes smoked per day[55]. Also, the same EPIC study noticed that passive smoking can increase the risk of pancreatic cancer by 50%[54].

The risk of developing pancreatic cancer increases with age[13,56]. Over 80% of pancreatic cancers develop between the ages of 60 and 80 years. Pancreatic cancer rarely occurs before the age of 40, and more than half of cases of pancreatic adenocarcinoma occur in those over 70. Pancreatic cancer affects men and women equally[1,56]. Studies in the United States have shown that pancreatic cancer is more common in the African American population than it is in the white population[57,58]. Some of this increased risk may be due to socioeconomic factors and to cigarette smoking[3,24,53,56].

According to an American Cancer Society study, obesity has been associated with increased mortality from cancer of the pancreas: risk of pancreatic cancer among obese (body mass index BMI $\geqslant$ 30) men and women compared with men and women of normal body mass index (< 25) was RR = 2.08[59]. A burden study published in 2011 estimated that, in the United Kingdom, around 12.8% of pancreatic cancers in men and 11.5% in women can attributed to overweight/obesity[9]. The recent meta-analysis has confirmed the hypothesis that both general and abdominal obesity are associated with increased pancreatic cancer risk[60]. Besides, physical inactivity has been linked with increased risk of cancer of the pancreas.

There is some evidence that intake of red or processed meat and high-temperature cooking may increase the risk of pancreatic cancer. In a large United Kingdom cohort study in 2016, low meat eaters (about 30%-45% lower mortality), as well as vegetarians and vegans (about 50% lower mortality) had lower mortality

for pancreatic cancer compared with regular meat eaters[61]. The EPIC study found no association between intakes of red and processed meat and pancreatic cancer risk, while poultry consumption was associated with an increased risk[62]. A recent meta-analysis of 11 prospective studies found a positive association between pancreatic cancer incidence and processed meat consumption[63]. But, some studies have not supported these findings[64], or have provided support for the association among men only[65]. On the other hand, frequent nut consumption is inversely associated with risk of pancreatic cancer in women[66,67]. A recent large nested case-control study in 2010 showed increased risk even at consumption of 60 g/d or more of liquor (spirits), and found no association with beer or wine[68]. Findings from the latest meta-analysis support that fruit and vegetable intake is associated inversely with the risk of pancreatic cancer[69]. Additionally, a summary review of meta-analytical studies showed that the major protective factor is increasing fruit or folate intake, with respective population preventable fractions of 0-12%[70]. In the Italian population, 11.9% of pancreatic cancers were attributable to a low adherence to Mediterranean diet[71].

Diabetes mellitus is linked with increased risk of pancreatic tumors[70,72]. Both type Ⅰ and type Ⅱ diabetes have doubled the risk of pancreatic cancer[72-74]. The pancreatic cancer burden study in the Italian population estimated that 9.7% of pancreatic cancers were attributable to diabetes[71]. The United States National Cancer Institute estimates that diabetes is associated with a 1.8-fold increased risk of pancreatic cancer, particularly in Hispanic men and Asians in comparison with whites and blacks[75]. Pancreatic cancer risk decreased with duration of diabetes, but a 30% excess risk persists for more than two decades after diabetes diagnosis[76]. Oral antidiabetics or insulin use were associated with a reduced risk of pancreatic cancer[75,76].

Some studies showed that *Helicobacter pylori* (*H. pylori*) infection is the major risk factors associated with pancreatic cancer, with estimated population attributable fraction of 4%-25%[70]. But, other studies did not observe an association of *H. pylori* infection with pancreatic cancer[77].

Patients with pancreatitis, especially the chronic or recurrent forms, had a moderate excess of pancreatic cancer risk[78]. About 4% of chronic pancreatitis patients developed pancreatic cancer[79]. It is estimated that 1.34% of pancreatic cancers are atributable to chronic pancreatitis, but for those who were under the age of 65 that risk was two times higher[80]. Patients with hereditary pancreatitis (rare, autosomal-dominant disease, usually occurs at a young age) have a risk that is 50-60 times greater than expected[81].

It is estimated that 5%-10% of pancreatic cancers are hereditary[9,52]. A family history of pancreatic cancer in a parent, sibling or child was associated with increased risk of pancreatic cancer[82]. People with at least two first-degree relatives (mother, father, brother, sister) with pancreatic cancer have almost double the risk of people without pancreatic cancer in their family[83].

There are many of inherited genetic disorders which are known to increase the risk for pancreatic cancer, including Lynch syndrome, Peutz-Jeghers syndrome, the Familial atypical multiple mole melanoma syndrome, Hereditary breast and ovarian cancer syndrome, Li-Fraumeni syndrome, Familial adenomatous polyposis, *etc*[83]. Individuals with mutations or deletion in such genes as PRSS1, K-ras, p16, p53, and BRCA2 have an increased risk of developing pancreatic cancer[84].

Some findings show a link between pancreatic cancer and previous cancers (cancer of the gallbladder, lung, stomach, uterus, breast, colon, *etc.*) and other conditions (Crohn's disease, gastric ulcer)[84-86].

Other potential risk factors include aspirin use, occupational exposure to certain pesticides, and dietary factors such as carbohydrate or sugar intake[70,86]. Most of pancreatic cancer risk factors are only weakly associated with the disease. Additionally, many people with pancreatic cancer do not have any one specific risk factor for it.

PREVENTION

There are no current screening recommendations for pancreatic cancer, so primary prevention is of utmost importance. A better understanding of the etiology and identifying the risk factors is essential for the primary prevention of this disease. Potentially modifiable risk factors include tobacco smoking, obesity, and diabetes mellitus, diet, alcohol consumption. So far, the best preventive strategy against pancreatic cancer is risk reduction, including lifestyle modification (smoking cessation, healthy weight, diet high in fruits and vegetables, regular exercises), and regular control of health issues[9,13,52,53].

Lifestyle modifications

Control of smoking offers the best available strategy for reducing the incidence of pancreatic cancer. It has been estimated that about 30% of pancreatic cancers could be prevented by prevention of smoking[9]. A European-wide prospective study in 2009 showed that risk is reduced to the levels of a non-smoker after just five years of cessation[54].

Epidemiological data show that dietary factors which are associated with increased risk of pancreatic cancer are meat, red meat in particular, and energy: preventive measures include recommendations for reducing the intake. Protection is mainly provided by means of a "prudent", well balanced diet, diet containing ample fruits and vegetables, nut consumption, vitamins (β-carotene, vitamin D and E), and more dietary compounds[26,87,88]. Also, it is necessary to limit the alcohol use, which is known to increase the

pancreatic cancer risk through development of chronic pancreatitis and cirrhosis[89].

Chemoprevention, with agents such as COX inhibitors and aspirin, could mean benefit for persons at high risk for pancreatic cancer and needs to be verified in future[90,91].

Chronic pancreatitis caused by heavy alcohol consumption or by an underlying inherited disorder is associated with a high risk of pancreatic cancer. Reduced alcohol use has been shown to reduce the risk of pancreatic cancer. Screening PRSS1 (Cationic trypsinogen gene) mutation as the mutation that causes hereditary pancreatitis is being performed for the development of a screening programme aimed at detecting pancreatic cancer at an early stage[92].

Patients with cystic neoplasms of the pancreas develop pancreatic cancer in about 60% to 70% of patients[93]. The complete extirpation of cystic neoplasms is now performed as a cancer preventive strategy[94].

Some risk factors such as age, gender, race, and family history cannot be modified. But, there is secondary prevention that might lower pancreatic cancer risk.

Screening and early detection

There is no reliable screening test currently available to screen the general population and detect pancreatic cancer early[95,96]. The research into the area of screening for pancreatic cancer has increased significantly in the past decade. Several screening tests were investigated to aid in the identification or earlier diagnosis of pancreatic cancer for the general population, including the blood markers for pancreatic cancer CA19-9, SPAN-1, CA-50, DUPAN-2, cell surface associated mucins (MUC), carcinoembryonic antigen, and heat shock proteins[10,97]. But these tests have not been well studied yet[98].

Although there are no screening tests currently available to screen the general population, researchers are working on developing effective screening tests that may be applied to groups of persons which have an increased risk of developing pancreatic cancer (such as persons with family history of pancreatic cancer, with abnormalities in certain genes - such as BRCA2, p16, HNPCC, and with histories of familial pancreatitis and Peutz-Jeghers syndrome, *etc.*)[98,99]. Also, question is which access points (stool, pancreatic juice, saliva and blood) could be appropriate to study these potential screening markers. For persons with high risk of pancreatic cancer (including patients with hereditary pancreatitis or for persons with family members with pancreatic cancer), some screening techniques (*i.e.,* endoscopic ultrasound and spiral computerized tomography) are promising but have not been fully evaluated[100]. In patients with hereditary pancreatitis (in PRSS1 mutation carriers) who have a higher risk of early onset of pancreatic cancer, screening can begin at age 40[92]. There is no consensus about when is screening initiating recommended for other persons. However, it remains an area of great interest[101].

CONCLUSION

Around the world significant efforts are being made in order to better understand pancreatic cancer. Detailed epidemiological analyses of pancreatic cancer trends and further analytical epidemiological researches will help guide future cancer control strategies.

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MINIREVIEWS

Galectin-3 and IL-33/ST2 axis roles and interplay in diet-induced steatohepatitis

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Abstract

Immune reactivity and chronic low-grade inflammation (metaflammation) play an important role in the pathogenesis of obesity-associated metabolic disorders, including type 2 diabetes and nonalcoholic fatty liver disease (NAFLD), a spectrum of diseases that include liver steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. Increased adiposity and insulin resistance contribute to the progression from hepatic steatosis to NASH and fibrosis through the development of proinflammatory and profibrotic processes in the liver, including increased hepatic infiltration of innate and adaptive immune cells, altered balance of cytokines and chemokines, increased reactive oxygen species generation and hepatocellular death. Experimental models of dietary-induced NAFLD/NASH in mice on different genetic backgrounds or knockout mice with different immune reactivity are used for elucidating the pathogenesis of NASH and liver fibrosis. Galectin-3 (Gal-3), a unique chimera-type β-galactoside-binding protein of the galectin family has a regulatory role in immunometabolism and fibrogenesis. Mice deficient in Gal-3 develop pronounced adiposity, hyperglycemia and hepatic steatosis, as well as attenuated liver inflammation and fibrosis when fed an obesogenic high-fat diet. Interleukin (IL)-33, a member of the IL-1 cytokine family, mediates its effects through the ST receptor, which is present on immune and non-immune cells and participates in immunometabolic and fibrotic disorders. Recent evidence, including our own data, suggests a protective role for the IL-33/IL-33R (ST2) signaling pathway in obesity, adipose tissue inflammation and atherosclerosis, but a profibrotic role in NASH development. The link between Gal-3 and soluble ST2 in myocardial fibrosis and heart failure progression has been demonstrated and we have recently shown that Gal-3 and the IL-33/ST2 pathway interact and both have a profibrotic role in diet-induced NASH. This review discusses the current evidence on

the roles of Gal-3 and the IL-33/ST2 pathway and their interplay in obesity-associated hepatic inflammation and fibrogenesis that may be of interest in the development of therapeutic interventions to prevent and/or reverse obesity-associated hepatic inflammation and fibrosis.

Key words: Galectin-3; Liver fibrosis; Interleukin-33; ST2; Nonalcoholic steatohepatitis

Core tip: Obesity-associated chronic low-grade inflammation (metaflammation) plays an important role in the pathogenesis of nonalcoholic steatohepatitis (NASH). Galectin-3 (Gal-3), a β-galactoside-binding protein, plays a regulatory role in metaflammation and tissue fibrosis. The Interleukin (IL)-33/ST2 pathway has a protective role in obesity and adipose tissue inflammation and promotes liver fibrosis. The characteristics of dietary-induced NASH differ in mice on different genetic backgrounds and Gal-3 and ST2 (IL-33R) knockout mice. In this report, we review current evidence on the roles of Gal-3 and the IL-33/ST2 pathway and their interplay in obesity-associated hepatic inflammation and fibrogenesis that may be of interest in the development of therapeutic interventions.

Pejnovic N, Jeftic I, Jovicic N, Arsenijevic N, Lukic ML. Galectin-3 and IL-33/ST2 axis roles and interplay in diet-induced steatohepatitis. *World J Gastroenterol* 2016; 22(44): 9706-9717 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9706.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9706

INTRODUCTION

Metabolism and immunity are closely connected and in the conditions of chronic overnutrition and low energy expenditure during obesity immune-mediated metabolic control are exerted in metabolic tissues, including adipose tissue and the liver. Metabolism and immunity share regulatory components that are yet incompletely understood[1]. Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), a severe form of NAFLD that can progress to liver fibrosis and cirrhosis, are regarded as a hepatic manifestation of metabolic syndrome[2]. The etiopathogenesis of NASH remains poorly understood, but existing data indicate that it is far more prevalent in obese individuals[3]. Obesity is characterized by increased adipose tissue mass, but also ectopic lipid accumulation and chronic low-grade inflammation in metabolic tissues[4]. The adipose tissue of obese individuals contains infiltrating immune cells and increased local generation of proinflammatory cytokines, as a result of the activation of stress kinases including c-Jun N-terminal protein kinases (JNK), IκB kinase (IKK) and protein kinase PKR, which in turn interferes with insulin signaling and triggers insulin resistance in adipose tissue, liver and skeletal muscle[5,6]. Therefore, obese individuals are at increased risk for developing both type 2 diabetes and liver diseases.

The diverse immune cell populations in the liver together with the inflammatory potential of nonhematopoetic hepatic cells have a central role in obesity-associated steatohepatitis and fibrosis[7]. Evidence suggests that cytokines are important mediators of diet-induced hepatic steatohepatitis, and the balance between proinflammatory Th1 and anti-inflammatory Th2 cytokines are changed[8]. Animal models of NAFLD/NASH are used for studies of pathogenesis of obesity-associated liver pathology including dietary models of experimental mice on various backgrounds with different susceptibility for the development of high-fat diet (HFD)-induced obesity and related metabolic disorders.

Galectin-3 (Gal-3), an evolutionarily conserved lectin that is produced by various cell types including immune cells and adipocytes, participates in immunometabolism[9-11]. Gal-3 has pro- and anti-inflammatory roles depending on the pathophysiological condition and tissue type. Gal-3 has the ability to bind to and dispose of advanced-glycation end-products and lipoxidation end-products[11,12]. Gal-3 is upregulated in adipose tissue in obesity and evidence including our own data demonstrates increased adiposity, hyperglycemia and enhanced inflammation in adipose tissues and pancreatic islets in Gal-3 knockout mice[13], which also develop accelerated and more severe pathology in experimental models of atherosclerosis and metabolically-induced kidney damage[14,15]. Interleukin (IL)-33, a member of the IL-1 superfamily of cytokines that is expressed by epithelial, endothelial and innate immune cells, functions as a traditional cytokine produced from living cells and as a nuclear factor regulating gene transcription. IL-33 can function as an "alarmin" - released following cell necrosis to alert the immune system to tissue damage or stress- and exerts its biological effects through binding to the its receptor, ST2[16] that is present on immune and nonimmune cells[17]. The IL-33/ST2 pathway is involved in the regulation of inflammation and remodeling during obesity and NASH[18]. Administered IL-33 enhanced accumulation of Th2 cells and promoted polarization of M2 type macrophages in adipose tissue, and reduced adiposity and glucose levels in obese diabetic (*ob/ob*) mice[17]. Recent data give evidence that IL-33 promoted expansion of $ST2^+$ T regulatory cells in adipose tissue during obesity and attenuated adipose tissue inflammation and insulin resistance[18]. IL-33 seems to have a hepatoprotective role in Concanavalin A-induced hepatitis and in ischemia-reperfusion injury[19,20]. Thus, the evidence that IL-33/ST2 signaling is an important regulatory pathway in

immunometabolic diseases are accumulating, but the role of this axis in obesity-associated liver pathology is scarce.

Gal-3 and IL-33 are two unrelated molecules, but recent studies point to the link between Gal-3 and soluble ST2 (sST2) in the pathophysiology of adverse myocardial remodeling and heart failure[21] and both molecules are regarded as prognostic markers in patients with acute myocardial infarction and acute or chronic heart failure[22,23]. Gal-3 promotes myocardial fibrosis, whereas myocardial fibrosis and hypertrophy are prevented through interaction between IL-33 and ST2[24]. The interaction between Gal-3 and the IL-33/ST2 pathway in liver fibrosis has not been studied, and reported data indicate that both pathways have a profibrotic role in obesity-associated hepatic fibrosis. Thus, Gal-3 and the IL-33/ST2 pathway may be potential therapeutic targets for treating or preventing liver pathology associated with obesity.

Here, we review the roles of Gal-3 and the IL-33/ST2 axis in the pathogenesis of liver metabolic disorders related to obesity and their interplay in diet-induced fibrotic NASH. These findings stem from our research of the effects of the genetic deletion of Gal-3 and ST2 in mice on relevant translational models of susceptibility to obesity-associated diseases.

PATHOGENESIS OF NASH

NAFLD is regarded as a hepatic manifestation of metabolic syndrome, and has increasing incidence worldwide that is in line with the increased prevalence of obesity and type 2 diabetes. NAFLD is a spectrum of liver diseases that encompasses simple steatosis, NASH and cirrhosis, and it is strongly associated with obesity and metabolic syndrome[25]. Visceral adipose tissue (VAT) from lean individuals and animals contain predominantly M2 type (alternatively activated) tissue resident macrophages that attenuate inflammation by secreting IL-10, whereas the VAT of obese individuals characterize the shift towards the M1 (classically activated) macrophages which secrete proinflammatory cytokines, such as TNF-α, that promote insulin resistance[26]. NASH and the consequential liver fibrosis represent major health problems without effective therapy[25,26].

Increased lipolysis in adipose tissue and increased plasma free fatty acids together with metaflammation are believed to promote lipid accumulation in hepatocytes leading to liver steatosis[27,28]. The pathogenesis of fibrotic NASH is described in a "two-hit hypothesis", wherein insulin resistance acts as "first hit" that leads to liver steatosis which renders hepatocytes more susceptible to a "second hit" which could involve inflammatory and oxidative stress mediators, lipotoxic fatty acids, cholesterol and ceramides[29]. In addition to this concept of the two-hit model, it is now appreciated that a more complex multiple parallel hits model is more likely responsible for the disease progression[30]. Current evidence suggest that lipotoxicity and oxidative stress represent the major mechanisms underlying hepatocyte dysfunction which initiates a cascade of steatonecrosis and inflammation. Additionally, deposition of fibrous tissue is the natural consequence of hepatocyte injury that is mediated by chronic inflammation[30,31]. NASH is characterized by intrahepatic accumulation of innate and adaptive immune cells through action of chemokines and cytokines that may also promote activation of hepatic stellate cells (HSCs) and their differentiation into myofibroblasts, the key players in the pathogenesis of liver fibrosis[32-34]. Intrahepatic immune cells and hepatic cells sustain chronic inflammation and by action of TGF-β and the IL-33/IL-13 pathway induce transdifferentiation of HSCs into myofibroblastic cells responsible for excessive extracellular matrix (ECM) deposition, mostly in the form of collagen[34-36] as illustrated in Figure 1.

MOUSE MODELS OF NASH

Experimental models that mimic the human NASH are needed to study causes and pathogenesis of this disease and to serve as adequate models for testing novel therapeutic strategies for this disease. Various options of animal models of NAFLD/NASH are used and are divided into genetic, dietary and combination models. Animals with naturally occurring mutations that inactivate key genes (*e.g.,* leptin gene in the ob/ob mice), animals with specific genetic manipulations, and animals subject to environmental and dietary variations are used for this purpose. Mice with different genetic backgrounds, knockout and transgenic mice, have been used in studies of obesity-related metabolic diseases[37,38]. The reported genotype-dependent differences in the susceptibility to developing fibrotic NASH could be associated with differential immune and inflammatory responses to metabolic danger molecules in these mice.

We have used C57Bl/6 and BALB/c mice, which are strains commonly used in studies on immunoregulation and HFD-induced metabolic disorders. The C57Bl/6 mice and BALB/c mice are regarded as prototypic Th1- and Th2-type mouse strains, respectively. Recent evidence indicates that the balance between M1/M2 macrophages and Th1/Th2 lymphocytes is of critical importance for the outcome of obesity-related metabolic disorders. The constitutive and HFD-induced differences in the distribution of immune cells in metabolic tissues exist in C57Bl/6 and BALB/c mice. We have recently reported inherent and HFD-induced differences in immunometabolic phenotype in these two mouse strains[39]. In response to HFD, the C57Bl/6 mice exhibited greater weight gain, higher glycemia, increased adiposity and higher prevalence of proinflammatory innate and adaptive immune cells in

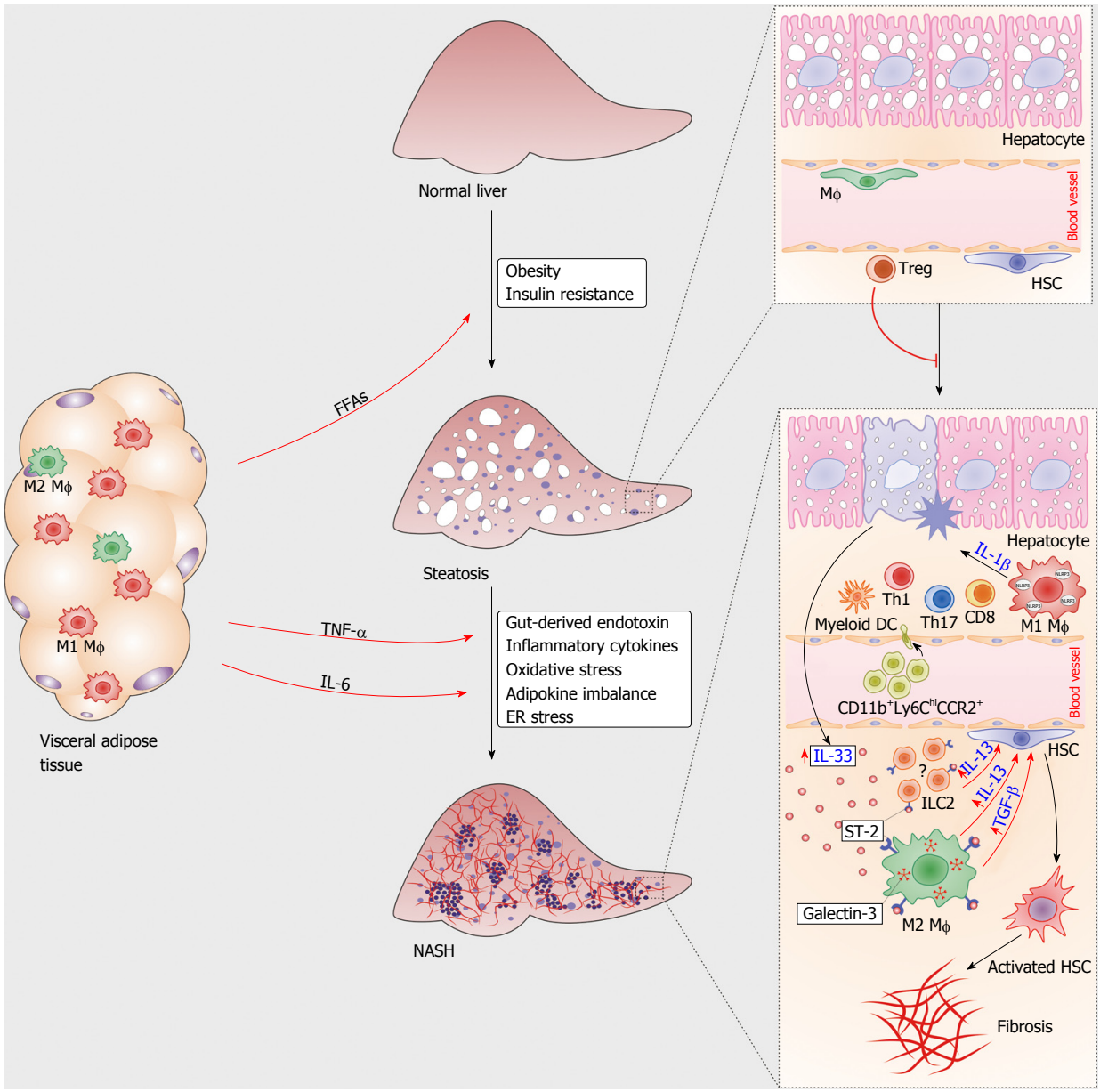

Figure 1 Immune cells in the pathogenesis of nonalcoholic steatohepatitis. During obesity, proinflammatory M1 macrophages and Th1-type lymphocytes infiltrated in the visceral adipose tissue mediate metaflammation that triggers insulin resistance. Increased amounts of free fatty acids (FFAs) released from adipose tissue accumulate in hepatocytes, causing liver steatosis. Liver regulatory T cells suppress metabolic inflammation. Multiple signals from visceral adipose tissue and gut polarize liver resident macrophages towards M1 type, promote chemotaxis of immune cells and hepatocyte injury. Damaged hepatocytes release IL-33, which promotes release of profibrogenic IL-13 and TGF-β from IL-33R (ST2)-positive macrophages. Liver resident innate lymphoid cells type 2 (ILC2s) might also respond to IL-33 by producing IL-13. Profibrogenic cytokines activate quiescent hepatic stellate cells, which transform to myofibroblasts, the key cells involved in the development of liver fibrosis.

VAT than the BALB/c mice. Th1-type mice on an HFD regimen were more susceptible to the development of visceral adiposity, liver inflammation and fibrosis, while the Th2-type mice were more susceptible to liver steatosis, which was associated with differential immune cell composition in adipose tissue and liver in this mouse strain[39]. In comparison to BALB/c mice, more numerous myeloid dendritic cells (DCs), proinflammatory macrophages and $CD11b^{+}Ly6C^{high}$ monocytes and $CD8^{+}$ T lymphocytes were found in livers of HFD-fed C57Bl/6 mice, together with higher levels of hepatic levels of IL-6, TNF-α and IFN-γ. C57Bl/6 and BALB/c mice differentially regulate the expression of genes related to lipid metabolism in liver in response to high-fat feeding, as HFD-induced marked liver steatosis and upregulated the hepatic LXRα and PPARγ genes in BALB/c mice. C57Bl/6 mice fed HFD developed liver fibrosis and had increased hepatic procollagen and TGF-β mRNA expression, and IL-33, IL-13 and TGF-β protein levels in liver homogenates, while diet-matched BALB/c mice had scarce collagen deposition in liver and lower levels of hepatic profibrogenic cytokines[39]. The representative images of HFD-induced liver pathology in C57Bl/6 and

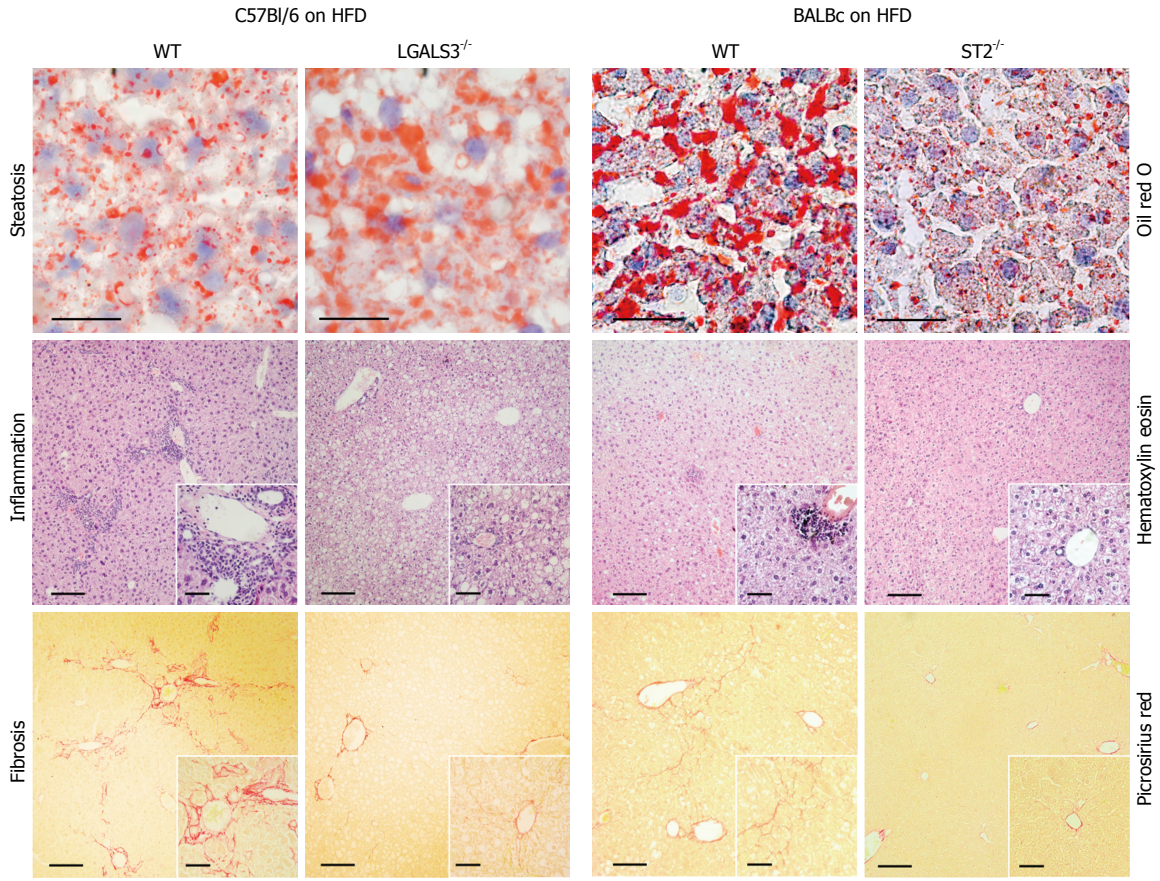

Figure 2 Galectin-3 and IL-33/ST2 axis in diet-induced steatohepatitis. Increased liver steatosis, but attenuated inflammation and fibrosis, in Galectin-3 knockout mice fed high-fat diet compared to diet matched C57Bl/6 wild-type mice (left panel). Decreased liver steatosis, inflammation and fibrosis in ST2 knockout mice fed high-fat diet compared to diet matched BALB/c wild-type mice.

BALB/c mice are shown in Figure 2. Strain-dependent differences in metabolic variables and composition of immune cells in metabolic tissues need to be considered in designing metabolic studies, particularly in studying obesity-associated fibrotic NASH.

GAL-3 IN HFD-INDUCED STEATOHEPATITIS

Galectins are evolutionarily conserved proteins that bind glycans, the carbohydrate structures broadly distributed on mammalian cells which can be altered by various pathological stimuli. There are 15 members of the galectin family which, *via* the carbohydrate-recognition domain (CRD), bind the cell surface β-galactosides and multiple terminal N-acetyllactosamine (LacNAc) sequences and cause intracellular signaling events involved in the regulation of various biological responses. The "galectin signalosome" has a role in many physiological and pathological conditions, and better understanding of its functions could lead to the development of novel therapeutic agents such as recombinant galectin proteins or specific galectin inhibitors.

Gal-3 has a unique "chimera-type" structure, having both lectin-like and CRDs, and can be present on the cell membrane, in the cytoplasm and the nucleus, and in extracellular spaces, including the systemic circulation[9]. Gal-3 recognizes endogenous glycans and modulates intracellular signaling pathways upon cell activation[40], proliferation[41,42] and apoptosis[43]. Gal-3 exerts important cell-cell and cell-ECM pro-adhesive roles[44,45], while also acting as a scavenger molecule for glucose and lipid adducts and in binding of microbial products, including endotoxin[46,47]. Gal-3 is expressed in innate and adaptive immunity cells and its production is altered in a variety of pathophysiological conditions, including autoimmune and inflammatory diseases, cancers and fibrotic disorders[13,48-50]. Gal-3 may have pro- and anti-inflammatory roles depending on the nature of the pathophysiological process, the type of tissue and the cellular localization.

Recent findings suggest an important regulatory role for Gal-3 in metabolic disorders, including obesity[13,51,52], diabetes[13,51], atherosclerosis[14], lipid-induced glomerular injury[15] and hepatic steatosis/inflammation[53]. The evidence so far, including our own data, indicates that gal-3 plays an important role in the regulation of adiposity, glucose metabolism, steatohepatitis and liver fibrosis in mice[13,53]. Obesity, *via* gain of ectopic fat and inducing metaflammation,

Table 1 Changes in mRNA levels of lipid metabolism and fibrosis-related genes in livers of LGALS3$^{-/-}$ and ST2$^{-/-}$ mice during high-fat diet-induced nonalcoholic steatohepatitis

Target gene	LGALS3$^{-/-}$ mice	ST2$^{-/-}$ mice
Abca-1	Upregulated	Unchanged
Cd36	Upregulated	Downregulated
LXR-α	Unchanged	Downregulated
PPARγ	Upregulated	Downregulated
Coll-α1	Downregulated	Downregulated
α-SMA	Downregulated	Unchanged
IL-33	Downregulated	Downregulated
IL-13	Downregulated	Downregulated
TGF-β	Unchanged	Unchanged

Abca-1: ATP-binding cassette transporter; LXR-α: Liver X receptor-alpha; PPARγ: Peroxisome proliferator-activated receptor gamma; Coll-α1: Procollagen alpha 1; α-SMA: Alpha-smooth muscle actin; IL-33: Interleukin-33; IL-13: Interleukin-13; TGF-β: Transforming growth factor-beta.

promotes insulin resistance, β cell failure and hepatic steatosis, thus representing the major risk factor for the development of type 2 diabetes and NAFLD.

The data regarding the role of Gal-3 in the pathogenesis of NAFLD are contrasting. Nomoto *et al*[54] have reported that Gal-3-deficient mice spontaneously develop steatosis at 6 mo of age. In addition, using the murine model of choline deficient, L-amino acid-defined diet - induced NASH that same group reported that ablation of Gal-3 led to a more pronounced steatosis and liver injury that could be related to and distinctive from the Gal-3 knockout mice on the CD1 background used in these studies[55]. In contrast, in a study by Iacobini *et al*[56], Gal-3 knockout mice were resistant to the development of steatosis and fibrotic NASH when fed an atherogenic diet. The authors demonstrated that proatherogenic HFD accelerated renal and aortic lesions, but attenuated NASH in Gal-3 knockout mice, which was accompanied by less fat deposition in liver and decreased oxidative stress. They also demonstrated that AGE/ALE levels and RAGE expression were decreased in the liver in spite of their increased circulating levels and that Gal-3 expressed on liver sinusoidal cells and endothelial cells has a major role in the uptake of these glucose and lipid adducts[56]. Gal-3 binds AGE/ALE *via* receptor-mediated endocytosis[57] and these harmful metabolic products are subsequently degraded by detoxifying enzymes[58].

NAFLD is strongly associated with obesity and meta-flammation, but the complex molecular mechanisms mediating development of liver steatosis and its progression to steatohepatitis and liver fibrosis are incompletely defined. The role of Gal-3 in the regulation of obesity-associated NASH has not been investigated. Therefore, we subjected wild-type (WT) and Gal-3 knockout mice on the C57Bl/6 background to obesogenic HFD (60% kcal from fat) for 24 wk and performed metabolic, histological, immunophenotypical and gene expression analyses in metabolic tissues[53]. We have demonstrated that the Gal-3-deficient mice fed HFD exhibit accelerated obesity and excess adiposity, hyperglycemia, insulin resistance, dyslipidemia and inflammatory changes in VAT and pancreatic islets. We have shown that obesity-associated hepatic lipid accumulation was uncoupled from the fibroinflammatory response in the liver in Gal-3 knockout mice in this experimental model[53]. In comparison to the WT mice, the HFD-fed Gal-3 knockout mice developed more pronounced liver steatosis which was accompanied by upregulation of hepatic FAS, PPAR-γ, Abca-1 and Cd36 mRNA expression, the lipogenic genes involved in fatty acid uptake and lipid synthesis[53] (Table 1). However, in obese Gal-3 knockout mice, liver injury, inflammation and fibrosis, and hepatic procollagen α1 and α-SMA mRNA expression were markedly lower compared to the WT mice on the same diet regimen, findings that were similar to the data reported by Iacobini *et al*[56]. The more pronounced hepatic fibro-inflammatory response induced by the obesogenic diet in the WT mice was associated with more numerous myeloid DCs and M1 macrophages (F4/80^{+}CD11c^{+}) infiltrated into the livers and higher hepatic F4/80, CD11c, NLRP3 inflammasome and IL-1β mRNA expression[53]. In contrast to the Gal-3 knockout mice, the WT mice on the obesogenic diet had increased percentages of CCR2^{+} proinflammatory monocytes (CD11b^{+}Ly6C^{hi}) in blood, bone marrow and liver and higher hepatic expression of CCL2[53]. HFD-fed Gal-3 knockout mice exhibited higher endotoxemia, but the hepatic TLR4 and CD14 NADPH-oxidase enzymes' mRNA expression was lower in comparison to the diet-matched WT mice. The obesity-driven greater steatosis was uncoupled with attenuated NASH in Gal-3-deficient mice, thus Gal-3 is involved in the progression of obesogenic diet-induced steatohepatitis in mice[53].

Furthermore, WT mice on HFD exhibited pronounced liver fibrosis accompanied by markedly higher hepatic expression of procollagen α1, IL-33 and IL-13 mRNA compared to Gal-3 knockout mice on the same diet regimen, while hepatic TGF-β mRNA expression was similar[53]. Gal-3 has an important profibrotic role and our data are consistent with the observation that Gal-3 disruption attenuated ECM production both *in vitro* in HSC cultures and *in vivo* in the model of carbon tetrachloride (CCl_4)-induced cirrhosis[59]. In this model, the disruption of the Gal-3 gene blocked TGF-β-mediated myofibroblast activation and procollagen expression, thus markedly attenuating CCl_4-induced liver fibrosis in mice.

Host-derived galectins may contribute to amplifying or attenuating of antimicrobial immune responses. Since Gal-3 directly interacts with the microflora and a variety of pathogenic bacteria, the contradictory results obtained when examining the role of Gal-3 in inflammation using Gal-3 knockout mice may at least in part be the consequence of different microbial populations and/or the involvement of

specific commensals in the disease pathogenesis under different experimental conditions[60]. Given the important involvement of the microflora in a variety of pathologies, including those of metabolic origin, a better understanding of the cross-talk between Gal-3 and commensal bacteria is necessary to clarify these issues.

Future studies will help to elucidate the role of Gal-3 in other metabolic tissues in the course of diet-induced obesity or aging. In particular, clarifying the mechanisms for the protective role of Gal-3 in pancreatic islets in the course of obesity would be of great importance. Obesity, diabetes, NASH and heart failure, and other diseases associated with inflammation in humans, are conditions that warrant better understanding of the role of Gal-3, especially in the light of current development of pharmacological inhibitors of Gal-3 for treatment of cancer and fibrosis.

IL-33/ST2 AXIS IN HFD-INDUCED STEATOHEPATITIS

The hallmarks of diet-induced steatohepatitis are the presence of liver steatosis, chronic inflammation in the liver and, under some circumstances, progression to liver fibrosis. Accumulated lipids in hepatocytes may promote the inflammatory response characterized by the increased infiltration of myeloid and lymphoid cells within the liver, activation of resident Kupffer cells and the secretion of pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6. Moreover, the most recent finding shows that IL-1 signaling promotes hepatic lipogenesis[61]. The role of other members of the IL-1 superfamily, including the IL-1 receptor antagonist, IL-18 and IL-33, together with IL-1 in obesity-associated liver pathology are incompletely defined.

IL-33 is a pleiotropic cytokine that binds to its plasma membrane receptor complex, comprising ST2 and the IL-1R accessory protein, and generally promotes Th2-type immune responses[62]. IL-33 exerts protective metabolic effects in obesity and atherosclerosis[17,63]. However, IL-33 promotes liver fibrosis through the activation and expansion of liver-resident innate lymphoid cells, which produce profibrotic IL-13[64]. The role of the IL-33/ST2 axis in obesity-associated liver pathology is not elucidated. We investigated the role of IL-33/ST2 signaling in the development of hepatic steatosis, inflammation and fibrosis using ST2-deficient mice on the BALB/c background which were placed on a long-term obesogenic HFD or high-fat high-fructose diet. The HFD-fed ST2-deficient mice exhibited increased weight gain and visceral adiposity compared with diet-matched WT mice. However, ST2 deletion markedly reduced hepatic steatosis, liver inflammation and fibrosis which was associated with lower expression of genes related to lipid metabolism in the liver. Innate immune cells, including $CD68^+$ macrophages and $CD11c^+$ DCs, were less numerous in HFD-fed ST2-knockout mice compared to WT controls. The HFD-fed ST2-knockout mice had less collagen deposition in the livers and lower numbers of profibrotic $CD11b^+Ly6C^{low}$ monocytes and Th17 cells in the liver, lower hepatic procollagen-α1, IL-33 and IL-13 mRNA expression, and lower serum levels of IL-33 and IL-13 compared with the diet-matched WT mice. Our findings suggest that the IL-33/ST2 axis has a complex role in obesity-associated metabolic disorders, as this pathway has a protective role in HFD-induced adiposity, but enhances liver steatosis, inflammation and fibrosis in NASH (Table 1). A very recent study[65] suggests that IL-33 treatment in HFD or methionine-choline-deficient diet (MCD)-fed C57Bl/6 mice attenuated hepatic steatosis, but aggravated hepatic fibrosis in a ST2-dependent manner. The reported effects of IL-33 on liver fibrosis are consistent with our data and the differential result regarding liver steatosis may be due to the genetic background of mice (*i.e.* BALB/c mice used in our experiments). Similar to our findings, the authors reported that the progression of NASH was associated with the increased mRNA expression level of IL-33 and ST2 in liver[65].

The significance of sST2 as a biomarker of liver fibrosis has been studied. The reported data demonstrate that the serum levels of sST2 were higher in patients with liver cirrhosis and hepatocellular carcinoma. These data suggest an important role of sST2 in the pathogenesis of hepatocellular carcinoma and liver cirrhosis as a possible marker of systemic inflammation. Further research is needed to evaluate the potential of sST2 as a prognostic marker in patients with liver fibrosis[66].

A recent study showed strong correlation between serum sST2 levels and fibrosis stages in patients with hepatitis B infection. It is suggested that serum levels of sST2 levels may be a reliable biomarker for evaluating the response to therapy for liver diseases causing fibrosis, including NAFLD[67].

GAL-3 AND IL-33/ST2 AXIS INTERACTION IN HUMAN PATHOLOGY

Gal-3 and the IL-33/ST2 axis are involved in myocardial remodeling. Gal-3 and sST2 are approved prognostic biomarkers that are involved in myocardial fibrosis and inflammation. Soluble Gal-3 is released by activated cardiac macrophages and stimulates proliferation of myofibroblasts and procollagen deposition. Higher concentrations of plasma Gal-3 are associated with myocardial remodeling, along with an increased risk of incident heart failure and mortality[68]. ST2 exists in two forms, a transmembrane receptor (ST2L) as well as a soluble decoy receptor (sST2). Through interaction between IL-33 and ST2L, myocardial fibrosis and hypertrophy are prevented. The sST2 acts as a decoy receptor that neutralizes IL-33,

so that the cardioprotective role of the IL-33/ST2L signaling pathway is lost, resulting in cardiomyocyte hypertrophy, apoptosis and fibrosis. Therefore, serum levels of sST2 are strongly predictive of adverse outcomes in patients with acute myocardial infarction or heart failure and significantly predict left ventricle remodeling. Thus, both sST2 and Gal-3 are reflective of fibrosis and cardiac remodeling, key events in heart failure[69].

Gal-3 and sST2 are promising biomarkers with additive diagnostic and prognostic value in the management of heart failure. Increased levels of Gal-3 indicate on-going fibrosis and reflect higher risk of heart failure, its severity and poor prognosis. The levels of sST2 are associated with the remodeling of left ventricle with significant prognostic value in heart failure. The head-to-head comparison of these two biomarkers in a large cohort of patients with a long-term follow-up revealed that sST2 is more important addition to established risk factors, so incorporation of the measurement of serum levels of sST2 into clinical practice is recommended[69]. Recently, serial testing for sST2 was shown to increase the prognostic information related to prediction of left ventricular remodeling and worsening of heart failure[70].

GAL-3 AND IL-33/ST2 AXIS INTERACTION IN NASH

In contrast to the opposite roles of Gal-3 and the IL-33/ST2 axis in myocardial remodeling, both Gal-3 and IL-33 exert profibrotic effects in liver fibrosis. Recently, evidence has demonstrated that Gal-3 is an important mediator in liver fibrotic models and that Gal-3 inhibitors protect against fibrotic disorders[71,72]. Tissue fibrogenesis is a complex process and newer data has pointed to the important cross-talk between cells of the immune system and tissue myofibroblasts in the evolution of liver fibrosis. Gal-3 is a molecule which can exert potent effects on multiple cell types, including myofibroblasts, and by altering the function of innate immune cells, such as macrophages[73]. In the CCl_4-induced model of liver fibrosis, Gal-3 ablation blocked HSC activation and collagen expression, as TGF-β failed to transactivate Gal-3-deficient HSCs, in contrast to the WT HSCs, suggesting that Gal-3 is required for TGF-β-mediated myofibroblast activation and ECM production. Gal-3 deletion reduced retention of TGF-β receptors at the cell surface and reduced phosphorylation and nuclear translocation of β-catenin, but had no effect on Smad2/3 phosphorylation[59]. Gal-3 represents a molecule that links macrophages, fibroblasts and the profibrotic response. Gal-3 mediates IL-4-induced alternative macrophage activation[74] and IL-4/IL-13 activated macrophages upregulate profibrotic genes and enhance fibrosis. Most recent data demonstrate a profibrotic role of IL-33 in a liver fibrosis model through ST2-dependent production of IL-13 by innate lymphoid cells (ILCs) that activate HSCs[64]. In our studies, we have addressed the issue of a possible regulatory role of Gal-3 in the newly described IL-33/ST2/IL-13 profibrotic pathway. Our recent data show that, in contrast to the Gal-3 knockout mice, HFD-fed WT C57Bl/6 mice had a higher number of hepatocytes that strongly expressed IL-33 and hepatic IL-13-expressing $CD11b^+$ myeloid cells, increased hepatic levels of IL-33 and IL-13 and increased mRNA expression of IL-33, ST2 and IL-13 in liver[53]. Moreover, IL-33 failed to induce ST2 upregulation and IL-13 production by Gal-3-deficient peritoneal macrophages *in vitro.* Similarly, exogenous IL-33 enhanced liver fibrosis in HFD-fed mice in both genotypes, albeit to a significantly lower extent in the Gal-3 knockout mice. This was associated with less numerous hepatic IL-13-expressing $CD11b^+$ cells. Whether Gal-3 is directly involved in the regulation of TLR4 and IL-13 mRNA expression remains to be elucidated. We and others[53,56] have shown that HFD increased TLR4 mRNA expression in livers of C57Bl/6 mice, which was markedly reduced in Gal-3-deficient mice[53]. A recent study also demonstrated that Gal-3 can be actively released by activated microglial cells and can bind directly to TLR4, thereby amplifying the typical TLR4-dependent proinflammatory response, including caspase-mediated inflammation[75].

IL-13 is produced by T lymphocytes, macrophages/DCs, mast cells and basophils and is induced by IL-33. The molecular mechanisms involved in Gal-3-dependent regulation of IL-13 mRNA expression are not known. We have shown that lower level of IL-13 mRNA in livers of HFD-fed Gal-3 knockout mice could be related to decreased hepatic IL-33 levels in these animals and the inability of Gal-3-deficient macrophages to respond to IL-33, which resulted in reduced percentages of $CD11b^+$ myeloid cells that expressed ST2 and IL-13 in livers[53].

The interaction of Gal-3 and the IL-33/ST2 pathway in diet-induced steatohepatitis is shown in Figure 3. These results provide evidence of a novel role for Gal-3 in regulating IL-33-dependent HFD-induced fibrotic NASH[53]. Thus, Gal-3 plays an important regulatory role in the newly described profibrotic IL-33/ST2/IL-13 pathway.

Further studies are necessary to clarify functions of intra and extracellular Gal-3 in liver fibrosis and in-depth research of the role of Gal-3 in IL-33 signaling is needed. A better understanding of the mechanisms regulating tissue fibrosis and targeted strategies to inhibit Gal-3 in the liver provide the rationale for the development of new therapeutic approaches for

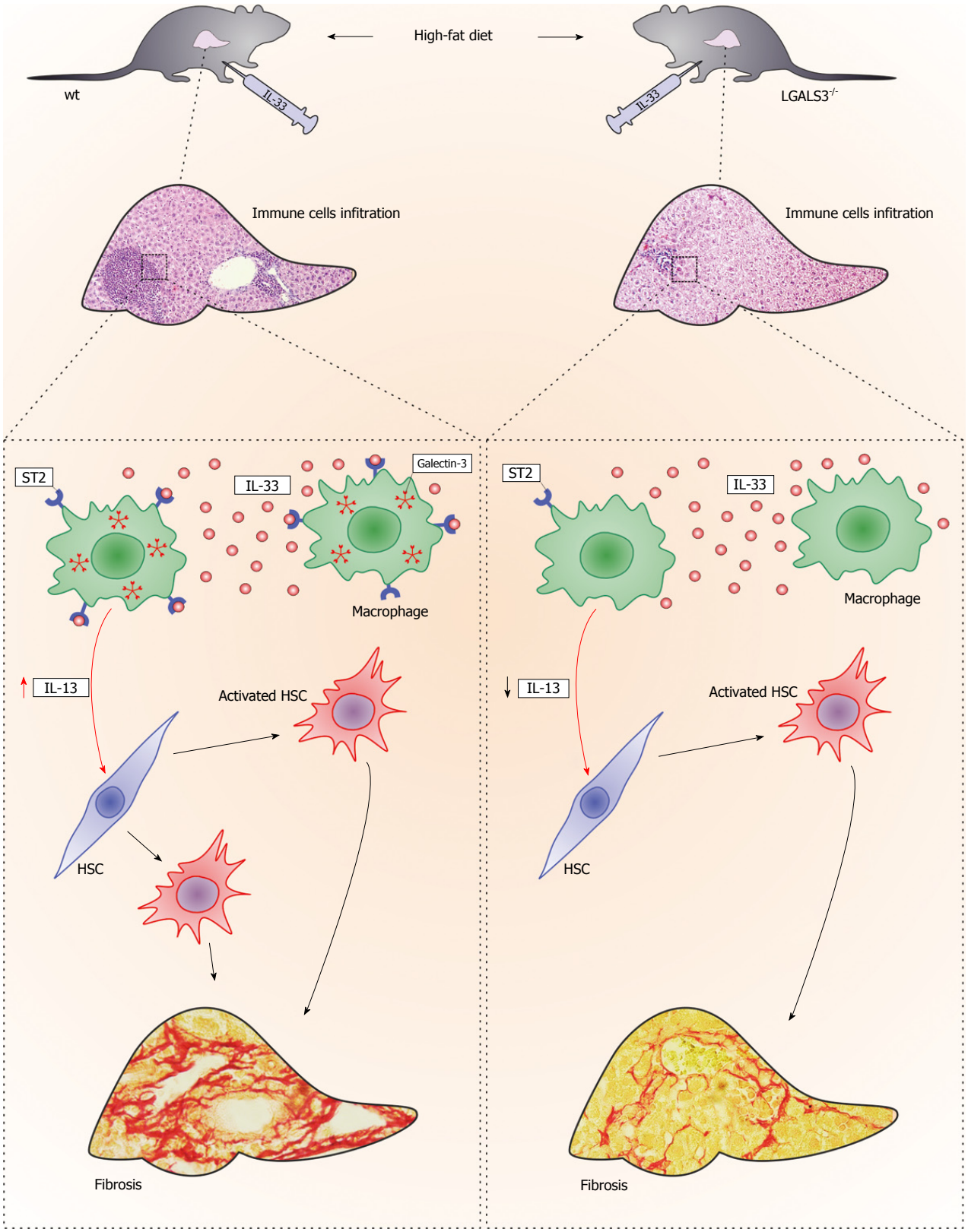

Figure 3 Galectin-3 and IL-33/ST2 axis interaction in diet-induced steatohepatitis. Administration of IL-33 *in vivo* enhanced high-fat diet-induced liver fibrosis in both genotypes of mice, although to a markedly lower extent in the galectin-3 knockout mice, which was accompanied by less numerous ST2-positive myeloid cells that express IL-13. Galectin-3 plays an important regulatory role in the newly described profibrotic IL-33/ST2/IL-13 pathway in hepatic fibrosis.

patients with liver fibrosis.

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MINIREVIEWS

Gene polymorphisms of pathogenic *Helicobacter pylori* in patients with different types of gastrointestinal diseases

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Abstract

Helicobacter pylori (*H. pylori*) is a kind of chronic infectious pathogen which can cause chronic gastritis, peptic ulcer, gastric cancer and other diseases. The genetic structure of the pathogenic genes of *H. pylori* varies largely, which contributes to the differences in virulence among various strains, and in clinical symptoms. Virulence genes of *H. pylori* can be categorized into three main classes: those related to adhesion and colonization, those related to gastric mucosal injury, and others. This review focuses on the relationship between genetic polymorphisms of the three classes of virulence genes of *H. pylori* and diseases. Most of the genetic polymorphisms of the main virulence factors of *H. pylori* are summarized in this paper.

Key words: *Helicobacter pylori*; Pathogenic gene; Polymorphism; Gastrointestinal disease

Core tip: *Helicobacter pylori* (*H. pylori*) is the causative agent of gastrointestinal diseases such as atrophic gastritis and peptic ulcers. Manifestations associated with chronic *H. pylori* infection vary considerably among distinct geographic regions and these differences have been attributed at least in part to polymorphisms of *H. pylori* genes, particularly those encoding virulence factors. There are several reviews for polymorphisms

of *H. pylori* genes. However, this is the first review to report the relationship between genetic polymorphisms and diseases. Virulence genes of *H. pylori* can be categorized into three main classes. This helps to understand the gene polymorphisms of pathogenic *H. pylori* in patients with different types of gastrointestinal diseases.

Chen YL, Mo XQ, Huang GR, Huang YQ, Xiao J, Zhao LJ, Wei HY, Liang Q. Gene polymorphisms of pathogenic *Helicobacter pylori* in patients with different types of gastrointestinal diseases. *World J Gastroenterol* 2016; 22(44): 9718-9726 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9718.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9718

INTRODUCTION

As one of the most common pathogens, *Helicobacter pylori* (*H. pylori*) is a Gram negative microaerophilic bacterium which is closely related to chronic gastritis, peptic ulcer disease, gastric cancer and mucosa associated lymphoid tissue lymphoma[1]. Even to the cases of non-gastrointestinal diseases, such as chronic cardiovascular disease, liver and biliary tract diseases and colorectal cancer, *H. pylori* also has a synergistic contribution[2]. In 1994 *H. pylori* was labeled as one of the first class cancer causing factors by the International Institute for Cancer Research[3]. Over half of the world's population are infected with *H. pylori* during their lifetime. With a small part of infected patients developing digestive diseases, gastric cancer, liver cancer and other tumors in severe cases, most of them have no dominant symptoms of infection[4,5]. The reason for high rate of *H. pylori* infection but low incidence or different severity of the disease happening is due to the genetic polymorphisms of *H. pylori*. The polymorphisms make the differences in virulence and pathogenicity of *H. pylori*, which lead to different clinical manifestations after *H. pylori* infection. The attention paid to the research about gene polymorphisms and pathogenicity of *H. pylori* is getting high. Pathogenicity of *H. pylori* related genes including vacuolating cytotoxin (vacA) and cytotoxin associated protein (CagA) has been well studied. Meanwhile some new pathogenic genes like homA and homB have also been identified. In this paper the study of gene polymorphisms of pathogenic *H. pylori* is summarized, aiming to provide suggestions for the research on the genetic polymorphisms of *H. pylori* in the future.

GENETIC POLYMORPHISMS OF *H. PYLORI*

The whole genome of *H. pylori* 26695 strain was sequenced in the early 1997. It was showed that there exist 1590 encoding sequences in the strain, representing 91% of chromosome DNA; in the non-coding region, gene internal sequences, non-coding repeats and stable RNA are included, which account for 6%, 2.3% and 0.7%, respectively. Of note, there are 499 unique nucleotides with high specificity in coding sequence[6]. In 2000 chip technology was used to detect 1643 genes of all the 15 strains of *H. pylori* in Stanford University for the first time, and it was found that conservative 1281 genes constitute the core part of the functional sequences, meaning that 12%-18% of genes are unique. *H. pylori* has been confirmed to be highly variable, which is one of its most important characteristics. High frequency mutations exist in nucleotide repeat sequences contained in multiple genes of the *H. pylori* genome. Nucleotide slippage of DNA template during replication promoted by *H. pylori* leads to open reading frame shift. By doing this genes can be switched between the modes of on and off easily.

H. pylori genome owns four unique characteristics in its spatial structure[7]. The first one is that about 1% of the genome encode a family consisting of 32 outer membrane proteins. Some members of this family, labeled as cell outer membrane pore proteins, probably are related to antibiotic sensitivity, which can result in multiple drug resistance of *H. pylori*, and development of chronic atrophic gastritis and other gastrointestinal diseases hard to cure. The second one is that more than 20 homologues contained in *H. pylori* genome are associated with DNA restriction and modification including type Ⅰ, type Ⅱ, type Ⅲ systems which are different among various species. That homologues may be involved in intra or extracellular DNA degradation, or in DNA recombination activation. The third one is that 46%-48% of specific *H. pylori* sequences were identified to be plastic. Many of the known plastic genes are not related to pathogenicity, but rather homologous with the restriction modified enzyme. The final one is that *H. pylori* gene production is associated with the biosynthesis of lipopolysaccharide and the system of DNA restriction modification. Meanwhile high frequency of homologous gene multimers and dinucleotide repeats can be found in cell surface associated proteins and enzymes.

In addition, *H. pylori* also shows polymorphisms during the genetic evolution. *H. pylori* genome adjusted itself to adapt to the environment under generation. Representing the adaptation to the host, polymorphisms of *H. pylori* were generated *via* a variety of common mechanisms, the most prominent of which is mutation and recombination. Besides, individuals from different subsets can also be storage hosts for each other, exchanging genes that are missing.

In summary, spatio-temporal distribution of *H. pylori* polymorphisms varies significantly. The variability not only lies in different strains but also can be detected in the same infected individual. Although high polymorphisms of *H. pylori* have been confirmed, the mechanism for high rate of *H. pylori* infection but low

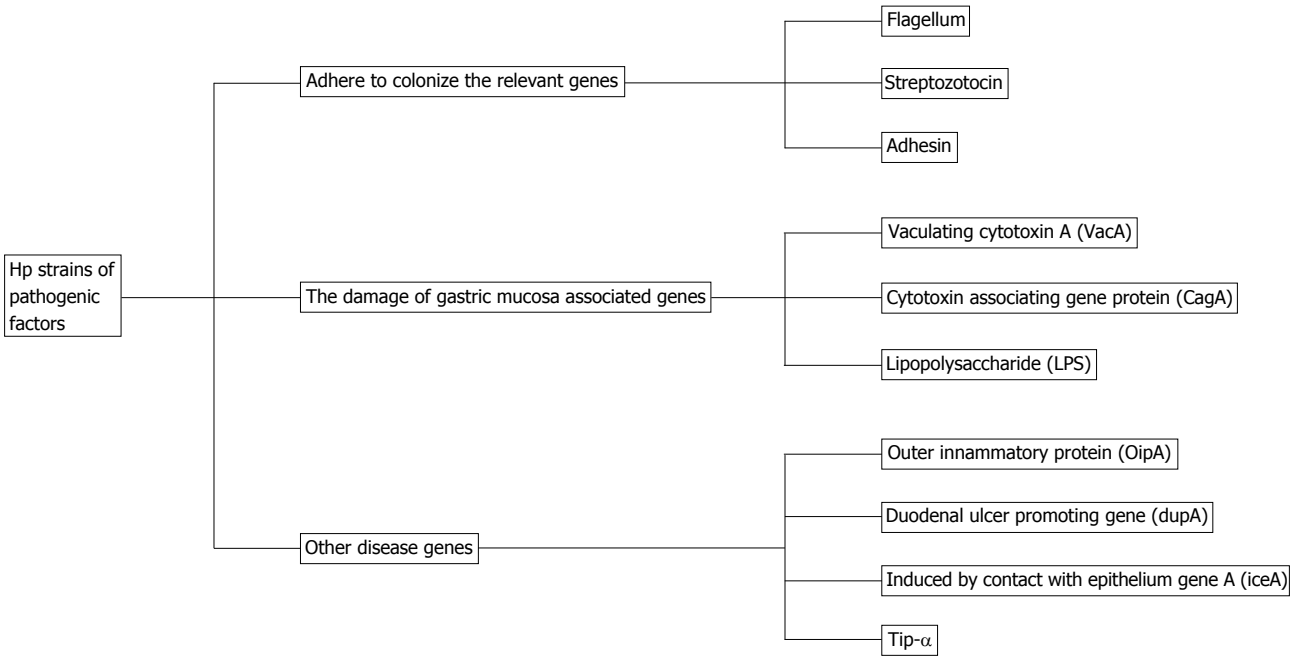

Figure 1 Pathogenic factors of *Helicobacter pylori* strains.

incidence of disease is still not well understood.

POLYMORPHISMS OF *H. PYLORI* VIRULENCE GENES

Pathogenic factors of *H. pylori* strains are categorized into three main classes: those related to adhesion and colonization, those related to gastric mucosal injury, and others. Specific pathogenic factors in the three classes are charted (Figure 1).

Genes related to H. pylori colonization

As a dynamic organ the flagellum is one of the most important virulence factors for colonization and pathogenicity of *H. pylori*. According to the report about the complete gene sequence of *H. pylori* 26695, more than 50 genes have been shown to play an important role in the biosynthesis, regulation and assembly of *H. pylori* flagella. Genes encoding *H. pylori* flagella mainly consist of *flaA*, *flaB*, *flgE*, *fliD*, *flhA*, *FlbA*, and *flgK*[8] . Flagellar filament of *H. pylori* is composed of multimeric flagellar structural proteins encoded by *flaA* and *flaB*. With 1533 gene sequences, *flaA* is transcriptionally regulated by the σ28 promoter[9]. The protein product of *flaA* contains 510 amino acids. Nucleotide sequence is highly conserved in the 1545 bp of *flaB*, the gene expression of which is controlled by the σ54 promoter[10]. And *flaB* encodes a peptide of 541 amino acids, 53.9ku. However, the restriction fragment length polymorphism analysis showed that a large number of gene mutations exist in *flaA* or *flab* (Figure 2)[11]. The diversity of *flaA* or *flaB* leads to the reevaluation on the sequence polymorphisms of *H. pylori* strains.

Blood-group antigen binding adhesion (BabA), encoded by *BabA*, can bind to fucosylated blood group antigen of Lewis B (Leb) or ABO (Figure 3)[12]. Inflammatory response caused by BabA positive *H. pylori* strains is severe. However, Leb is not expressed in all of huamn gastric mucosal cells. In case of Leb deficiency, the interaction between BabA and Leb does not account for the adhesion of *H. pylori*. *BabA* has three genotypes: *babA1*, *babA2* and *babB*. The former two are highly homologous. Compared to *babA1*, an additional 10 bp sequence is inserted into the signal peptide of *BabA2*, forming the transcription initiation codon. Only the genotype of *babA2* has function. On the contrary, *babA1* loses function due to the incomplete signal peptide. *babA1* deficiency has no effect on the binding between *H. pylori* and Leb. Thus it is the active BabA encoded by *babA2* that strengthens inflammation response of the stomach, and induces duodenal ulcer or gastric cancer.

H. pylori outer membrane protein AlpB, is involved in *H. pylori* adhesion, especially in the first step for *H. pylori* colonization. In the stomach, *H. pylori* adhere to host cells, inducing cytokines to cause mucosal injury. Polymorphisms of *AlpB* exist not only in the adaptive strains, but also in the strains colonizing in the human body. Presumably, *AlpB* varied to adapt to specific microenvironment of the stomach, which makes the polymorphisms of this domain.

Encoded by *sabA*, sialic acid binding adhesin A (SabA) is very important in the outer membrane protein family of *H. pylori*. *SabA* can recognize X Lewis (LeX), an antigen of the human gastric epithelial cell surface. *SabA* does not correlate with anti-LeX antibodies in human[13]. With polymorphisms, *SabA* is expressed selectively during *H. pylori* replication to

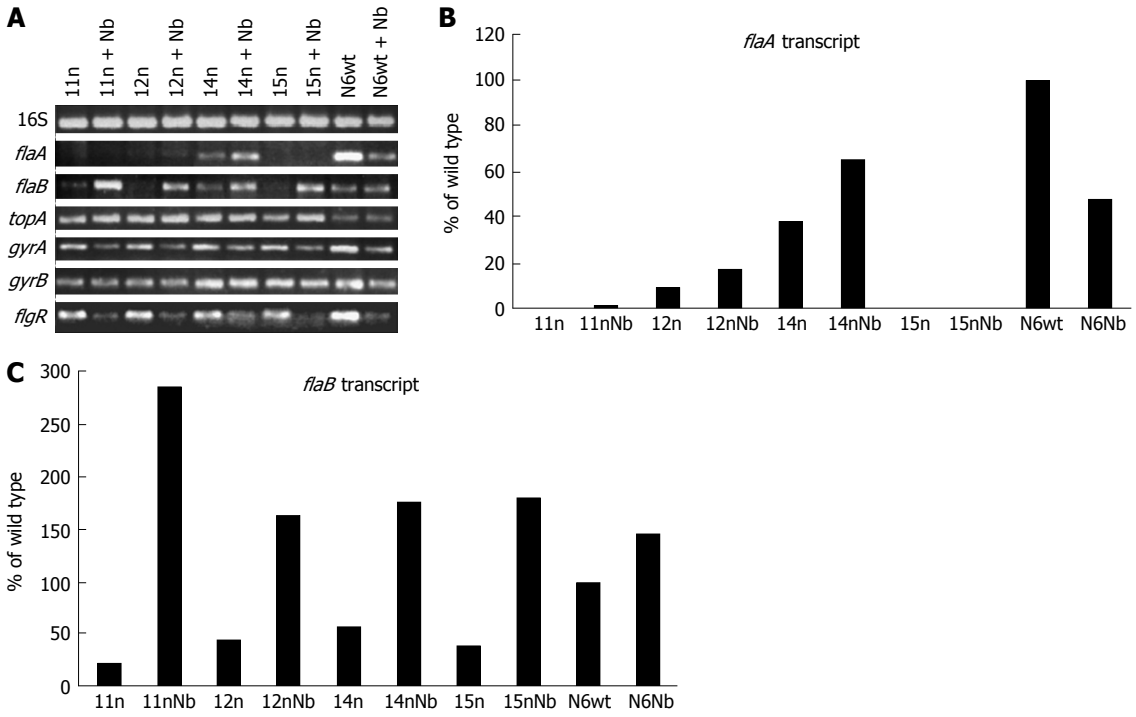

Figure 2 Analysis of *flaA* promoter spacer mutants reveals dependency of promoter activity on spacer length and supercoiling. A: Detection of *flaA*, *flaB*, *topA*, *gyrA*, *gyrB*, and *flaR* transcript abundance by RT-PCR for wild-type *flaA* promoter (13n), 11n 12n, 14n, and 15n spacer mutants, grown with and without novobiocin. Result of RT-PCR of 16S rRNA is shown to permit comparisons of mRNA quantity. Quantitation of *flaA* and *flaB* transcript levels (AnalySIS software, Soft Imaging Systems) is shown in (B) and (C).

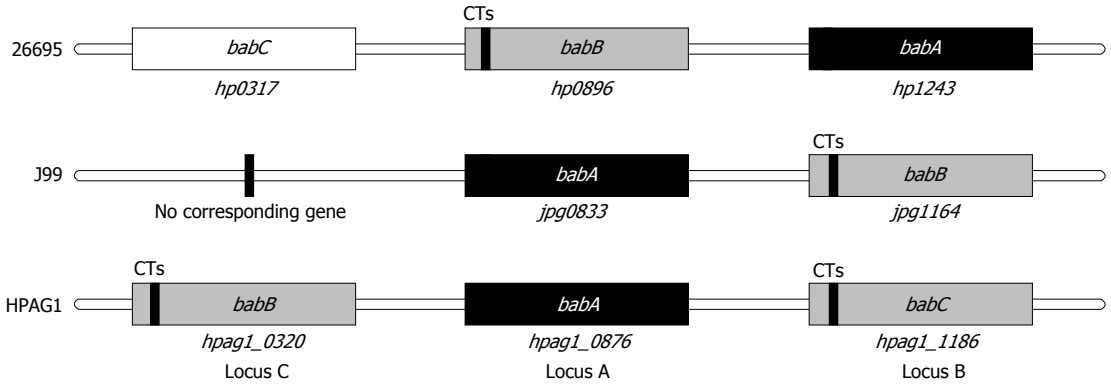

Figure 3 Genomic locations of the *babA*, *babB*, and *babC* genes in strains J99, 26695, and HPAG1. CT: CT dinucleotide repeats.

adapt to the host and avoid immune attack[14]. When its level is high, saliva LeX correlates with *H. pylori* colonization in the stomach[15]. The frame shift of the CT repeat in the 5′ upstream region makes phase mutation of *SabA* (variation phase) to regulate its expression.

β3N-acetylglucosamine T5 (β3GnT5), a kind of the transferase of N-acetylglucosamine (GlcNAc), is the key for the Lewis antigen synthesis. Synergy between β3GnT5 and cagPAI leads to an elevation of the adhesion ability of *H. pylori*. CagA and CagE in both β3GnT5 and cagPAI positive *H. pylori* strains stimulate β3GnT5 and Lewis antigen and result in strong colonization of SabA in epithelial cells[16].

Genes related to gastric mucosal injury

VacA is one of the most important virulence factors in the pathogenesis of *H. pylori*. *VacA* exists in all of the *H. pylori*, but its virulence region is not expressed in all individuals. Western blot analysis with VacA antibody showed that virulence of *VacA* depends on the middle region (M) and signal area (S) of its structure. VacA with virulence mainly interferes phenotype and structure of epithelial cells, inducing cellular barrier dysfunction, inflammation changes, and vacuolization[17,18]. Binding to cell surface specific receptors, VacA enters cells by endocytosis, then affects cellular membrane transport, results in swelling, vacuolization, and apoptosis in the end[19]. In 2007, the

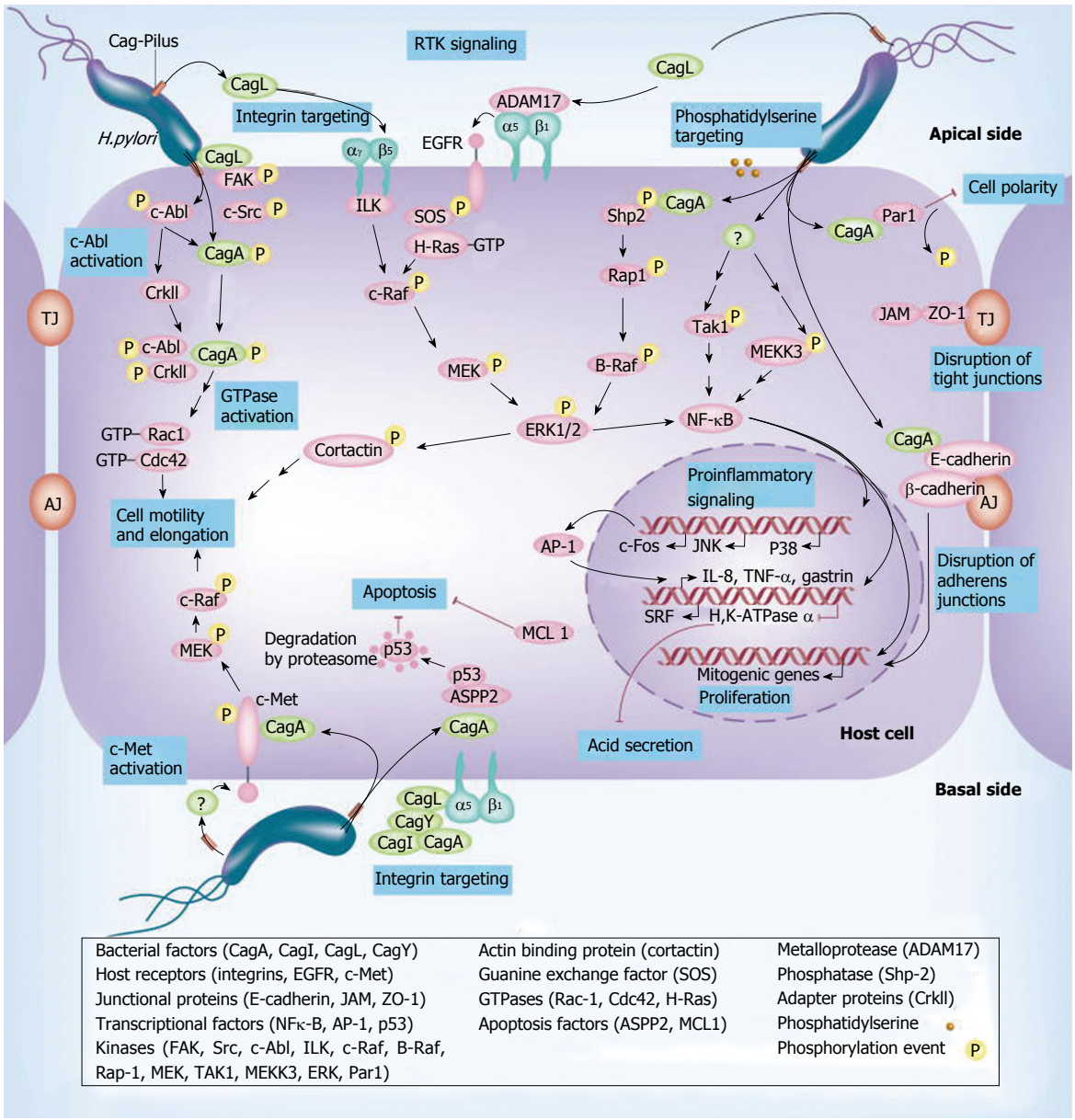

Figure 4 Model for the role of *Helicobacter pylori* T4SS effector proteins in host cell interaction and disease-associated signal transduction.

third region with polymorphism was found in *vacA*[20], named intermediate region (i), which is between the area of S and M. *vacA* is divided into two subtypes of i1 and i2 by the difference of I region (Figure 2). Genetic linkage exists between i region and s or m area. The s1/m1 or s2/m2 displays the genotype of i1 and i2, respectively, while the s1/m2 displays genotype of i1 or i2. The relationship between i genotype and disease is region dependent[21,22]. Both the S1 and S2 are not pathogenic. Virulence of the M1 is stronger than that of the M2 under the same condition. The i1 has virulence, but the i2 is of weak virulence or avirulence.

Another virulent factor of *H. pylori* is cagA. When cagA is transported into the epithelial cells of host gastric mucosa by type IV secretion system encoded by CagPAI, a tyrosine residue on it becomes phosphorylated. Interacting with a variety of proteins of the host cells, CagA amplifies, causing a change in the morphology of gastric epithelial cells (Figure 4)[23,24]. In recent years, polymorphisms of *cagA* was revealed to encode four proteins like A, B, C, and D, which play a key role in cytoskeletal rearrangement, infiltration and cell proliferation.

Gene cluster of cytotoxin associated gene pathogenicity island (cagPAI), is a kind of typical structure in the bacterial chromosome mainly encoding bacterial virulence related structure proteins and metabolic products. With a length of 37 kb, cagPAI consists of about 30 genes, and is one of the main hypervariable regions of the genome (Figure 5)[25].

cagPAI encodes type Ⅳ secretion system (TFSS) of *H. pylori*. With a piliform appearance, TFSS is a macromolecule across both inner and outer membranes of *H. pylori*, forming a transmembrane channel.

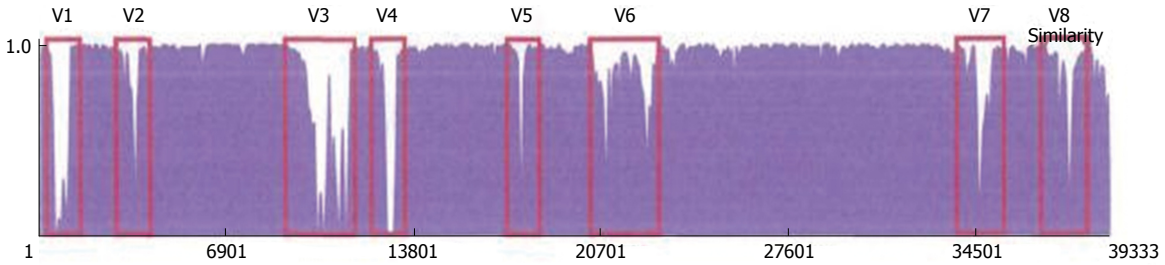

Figure 5 Eight variable regions in cagPAI sequences of Chinese *Helicobacter pylori* strains.

Pathogenic *H. pylori* can be transported into host cells through the channel, causing cellular lesions, inducing cytokines such as interleukin 8 (IL-8), and promoting inflammation[26]. In the West cagpaI and cagA are used to evaluate the virulence of *H. pylori*. But in Southeast Asia 90% of the people are both cagpaI and CagA positive with different clinical symptoms. The relationship between cagpai high variability and disease was studied (Figure 5). The result showed that most of cagpaI sequences in *H. pylori* strains worldwide are conservative. With few large fragment deletions, only a few genes of cagPAI are diverse, and even variations are meaningless to its virulence[27].

Hom A and *Hom B* are newly discovered to be closely associated with gastric cancer development. Like genes encoding the outer membrane proteins of *H. pylori*, both *Hom A* and *Hom B* are present with allelic variations, but they are different in the very important intermediate region of 300 bp. The highly polymorphic region of *HomB* is located from 750 bp to 1050 bp, while in *homA* it is in the area from 720 bp to 980 bp[27]. In addition, variability of *HomB* allele is higher than that of *HomA*. The sequence analysis of the nucleotides and amino acids showed copy number and site polymorphisms in *Hom A* and *Hom B,* which affected their function. Besides, recombination in *Hom A* and *Hom B* regulated DNA replication or *HomA*/*HomB* conversion[28-30]. *Hom A* and *Hom B* consist of six allelic variants from AI to AVI. Among them, AI and AII are the main[27]. As new virulence factors of *H. pylori*, HomB can be used to distinguish strains from duodenal ulcer and gastric cancer[31,32]. HomB is closely related to the severity of inflammation or atrophy of the gastric mucosa[32,33]. Analysis[34] of 289 strains of *H. pylori* from the United States and Columbia showed that 71.9% of the strains in gastric cancer was HomB positive, which was significantly higher than that in strains from duodenal ulcer (52.1%). The expression of *cagA*, *HomA* and *HomB* in 138 strains of *H. pylori* from Iran was studied[35]. It was found that 78% of the strains in gastric cancer were HomB positive, which was significantly higher than that in peptic ulcer (20%) or gastritis (43%). The positive rates of cagA in strains in gastric cancer, peptic ulcer or gastritis were 68.3% 54.8%, and 51.4%, respectively, without a significant difference. The above results suggested that compared to cagA, HomB is more likely to be a marker for distinguishing gastric cancer and duodenal ulcer. However, it is controversial[36,37].

Other pathogenic genes

As one of the 32 outer membrane proteins, pro-inflammatory outer membrane protein A (OipA) is closely related to the clinical symptoms of *H. pylori* infection, bacterial colonization density, and severe neutrophil infiltration development. OipA can induce the activation of adhesion kinase center and promote cytoskeletal rearrangement[38]. The expression of *OipA* is regulated by the "slip chain mismatch" mode which involves frame shift of "CT" repeat in the signal region of *OipA*. OipA overexpression in cagPAI deleted strains induced IL-8 in gastric mucosal epithelial cells three times higher than that in stains deficient of both. Expression of *OipA* makes it functional and is related to the clinical symptoms and the severity of the disease.

Duodenal ulcer promoting gene (dupA) is a kind of factor encoding type Ⅳ secretion system, and it is located in the plastic zone of *H. pylori* gene. Homogenous to virB4, *dupA* owns two open reading frames of jHp918 and jHp917[39]. *dupA* in *H. pylori* exposes the infected patients especially from East Asia to duodenal ulcer with a high rate, but to atrophic gastritis and gastric cancer with a low rate. It was showed that related to IL-8 up-regulation in the gastric mucosa, *dupA* can promote duodenal bulb ulcer in *H. pylori* infected patients, and suppress atrophic gastritis and gastric cancer, which is obvious in strains from both Asian and Western populations.

After CagA and VagA, mucosal contact inducible factor (iceA) was revealed as a new pathogenic factor in recent years. Located in the region between the conserved *cysE* and *HpyIM* of *H. pylori*, iceA1 was found to consist of two subtypes of iceA1 and iceA2 by multi strain sequence analysis. The correlation between iceA gene and gastric mucosal lesions has been studied[40-44]. By meta-analysis and statistical analysis, *iceA1* gene was found to mainly exist in duodenal ulcer patients. There is no significant correlation between the distribution of *iceA2* and peptic ulcer disease. It appears that two subtypes of iceA correlated differently with peptic ulcer disease[45]. It was recently found that the ratios of the iceA subtypes in disease are not the same in different countries or regions. Successful overexpression of the two indicated that positivity for

Table 1 The relationship between gastrointestinal diseases and *Helicobacter pylori* virulence factors

				VacA							IceA		
	HomA	HomB	DuPA	VacAS2	VacAS1	VacM2	VacM1	Vaci2	Vaci1	CagA	IceA1	IceA2	OiPA
Chronic gastritis	+	++++	++	+	++++	+++	++	+	++++	++++	++	+	-
Dudenal ulcer	+	++++	++	-	++	++	+	+	++	++	+	+	++
Gastric ulcer	+	++++	+	+	+	+	+	-	-	+	+	+	++
Gastric cancer	+	++++	++	-	+	+	++	+	+++	++	+	+	++++

+: Incidence of disease, 0-20%; ++, 20%-40%; +++, 40%-60%; ++++, 60%-80%; +++++, 80%-100%.

both genotypes of *iceA1* and *iceA2* may be a marker of *H. pylori* induced acute inflammatory response in the gastric mucosa[46-48]. The purified protein can also be used for the auxiliary diagnosis of related clinical diseases.

Tumor necrosis factor α induced protein (Tip-α) is a newly found soluble toxin, encoded by the open reading frame of 0596 gene in *H. pylori*. With a length of 519 bp, *Tip*-α encodes a peptide of 173 amino acids[49]. Dimer through a pair of disulfide bonds (Cys25-Cys25 and Cys27-Cys27) in the N terminus is the active form of Tip-α which can greatly induce IL-1b, IL-8, and TNF-α, mediated by NF-κB, to promote the inflammatory response of the host and the occurrence and development of the tumor together with a series of gastrointestinal diseases[50].

RELATIONSHIP BETWEEN *H. PYLORI* PATHOGENIC GENES AND GASTROINTESTINAL DISEASES

The polymorphisms of virulence genes of *H. pylori* are closely related to the clinical outcome of infection, especially in cases of positivity for *VacA*, *cagA*, *SabA* or *iceA*, different genotypes of which result in different types of gastrointestinal diseases (Table 1). It was showed that 80%-100% of *HomeB* positive ones suffered from various gastrointestinal diseases, which indicated that *HomeB* can be treated as one of the main newly found virulence genes; belonging to the high virulence genes, *CagA*, *VacA* and *DupA* mainly cause gastritis disease, duodenal ulcer and gastric cancer, while *OiPA* mainly causes gastric and duodenal ulcers and gastric cancer. In addition, the research found that[51] the progression from atrophy gastritis to cancer is regulated by *H. pylori* in the host through changing dominant microflora and thus regulating gastric epithelial cell related signal transduction, metabolism and cancer development related tumor suppressors[52]. At the same time, the micro-evolution is very common in China, leading to changes of gastric flora. Strains of *H. pylori* with more than two kinds of genotypes exist in 99% of the hosts, resulting in repeated drug resistance and recurrence (Table 1)[53].

CONCLUSION

The development of *H. pylori* associated diseases is related to the virulence, the amount of pathogenic bacteria, and the immunity of oneself. The polymorphisms of pathogenic genes make the differences in the virulence of various strains, which closely correlates with the outcome of clinical infection with no clinical symptoms, ulcers, inflammation and even tumors following, and non-gastrointestinal system disease as the result. With the development of molecular biology and proteomic technology, some new pathogenic factors of *H. pylori* have been found, resulting in the amplified mechanism underlying the pathogenesis. However, pathogenic analysis of *H. pylori* is very difficult due to the presence of unknown pathogenic genes, involvement of multiple factors in diseases induced by *H. pylori* infection, and the combination of many types of strains in the same patient. Thus further study on the relationship between genetic polymorphisms of *H. pylori* and its associated clinical diseases should be conducted to fully illustrate the pathogenicity of *H. pylori*.

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ORIGINAL ARTICLE

Basic Study

Infliximab does not increase colonic cancer risk associated to murine chronic colitis

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Author contributions: Lopetuso LR did most of research and writing; Petito V performed samples processing and *in vitro* analysis; Arena V performed histological and tumor analysis; Zinicola T, Graziani C, Gerardi V assisted with writing; Caristo ME and Poscia A performed the statistical analysis; Cammarota G, Papa A and Cufino V assisted with the editing; Sgambato A, Gasbarrini A and Scaldaferri F conceived the original concept, assisted with the research and performed the critical revision of the manuscript; Lopetuso LR, Petito V, Gasbarrini A and Scaldaferri F contributed equally to this work.

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Abstract

AIM

To explore the influence of Infliximab (IFX) on cancer progression in a murine model of colonic cancer associated to chronic colitis.

METHODS
AOM/DSS model was induced in C57BL/6 mice. Mice were injected with IFX (5 mg/kg) during each DSS cycle while control mice received saline. Body weight, occult blood test and stool consistency were measured to calculate the disease activity index (DAI). Mice were sacrificed at week 10 and colons were analyzed macroscopically and microscopically for number of cancers and degree of inflammation. MTT assay was performed on CT26 to evaluate the potential IFX role on metabolic activity and proliferation. Cells were incubated with TNF-α or IFX or TNF-α plus IFX, and cell vitality was evaluated after 6, 24 and 48 h. The same setting was used after pre-incubation with TNF-α for 24 h.

RESULTS
IFX significantly reduced DAI and body weight loss in mice compared with controls, preserving also colon length at sacrifice. Histological score was also reduced in treated mice. At macroscopic analysis, IFX treated mice showed a lower number of tumor lesions compared to controls. This was confirmed at microscopic analysis, although differences were not statistically significant. *In vitro*, IFX treated CT26 maintained similar proliferation ability at MTT test, both when exposed to IFX alone and when associated to TNF-α.

CONCLUSION
IFX did not increase colonic cancer risk in AOM-DSS model of cancer on chronic colitis nor influence directly the proliferation of murine colon cancer epithelial cells.

Key words: Inflammatory bowel disease; Ulcerative colitis; Colorectal cancer; Infliximab; AOM-DSS model; Cancer on chronic colitis

Core tip: We report our results on the potential role of Infliximab on cancer progression in AOM-DSS murine model of colorectal cancer associated to chronic colitis. AOM/DSS model was induced in C57BL/6 mice. Mice were injected with Infliximab during each DSS cycle while control mice received saline. Mice were sacrificed at week 10 and colons were analyzed macroscopically and microscopically for number of cancers and degree of inflammation. MTT assay was performed on CT26 to evaluate the potential influence of Infliximab (IFX) role on metabolic activity and proliferation. This study demonstrates that beside its well-known healing capacity, IFX does not increase proliferative cancer cells ability and colorectal cancer risk in AOM-DSS model of tumor on chronic colitis.

Lopetuso LR, Petito V, Zinicola T, Graziani C, Gerardi V, Arena V, Caristo ME, Poscia A, Cammarota G, Papa A, Cufino V, Sgambato A, Gasbarrini A, Scaldaferri F. Infliximab does not increase colonic cancer risk associated to murine chronic colitis. *World J Gastroenterol* 2016; 22(44): 9727-9733 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9727.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9727

INTRODUCTION

Inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing inflammatory disorder of the digestive tract resulting from a loss of homeostasis between the intestinal immune system and the gut microbiota in genetically-predisposed individuals[1]. Inappropriate mucosal immune responses, due to disruption of the epithelial barrier separating microorganisms from underlying tissues and/or dysregulated tolerance to the intestinal microbiota, likely contribute to the development and perpetuation of IBD[2-5]. In this scenario, long history of colonic IBD is associated with colonic cancer progression[6].

Colorectal cancer (CRC) is an important worldwide medical problem as it is the third most commonly diagnosed serious form of cancer in men and in women and represents the third leading cause of cancer death for men and for women and the second leading cause of cancer mortality overall[7]. Sporadic cases are the most frequent. UC patients have a 20-fold higher risk to develop CRC compared to general population. This risk is higher especially in pancolitis: 30% of patients with pancolitis develop CRC after 30 years of disease[7,8]. In addition, UC patients with CRC have an overall poor prognosis and limited therapeutic options are available. Mechanistically, CRC in chronic colitis has a different pathogenesis compared to sporadic or familial cancer[6]. Experimental observations provide full support for the role of inflammation in IBD-related colorectal carcinogenesis. The administration of agents that cause colitis in healthy or genetically modified rodents accelerates the development of CRC[9]. Mice genetically predisposed to develop IBD also develop CRC, especially in the presence of microbial colonization[10]. Although there is little doubt that chronic inflammation promotes colon cancer, the mechanisms involved are unclear. Tumor Necrosis Factor (TNF)-α and other cytokines involved in the development of chronic gastric inflammatory conditions play a key role in triggering early epithelial alterations observed in intestinal metaplasia and in promoting the progression to epithelial dysplasia[11,12]. Moreover, proinflammatory mediators represent critical factors in promoting the growth of neoplastic lesions, inducing epithelial cell proliferation and neoangiogenesis[12,13].

In this setting, anti-TNF-α drugs, such as Infliximab (IFX), represent a crucial therapeutic tool for modulating the immune response and the clinical course in IBD[14].

IFX is a monoclonal chimeric antibody. Its clinical efficacy and ability to provide mucosal healing in CD as well as in UC are well demonstrated[14]. However, there are concerns regarding the impact of TNF-α blockade on the incidence of malignancy. In particular, while the curative role of IFX on chronic intestinal inflammation is supposed to reduce CRC risk, a secondary neoplasm is one of the most feared sequelae of the immune system manipulation in general[15,16].

The combination of Azoxymethane (AOM) with exposure to dextran sodium sulphate (DSS) in mice is a well-known CRC model for studying the progression of CRC on chronic colitis and related mechanisms of action. In the present study, we explored the influence of IFX on cancer progression in AOM-DSS murine model of CRC cancer associated to chronic colitis. We showed that IFX significantly reduced disease activity index and body weight loss, preserving colon length at sacrifice. Histological analysis revealed an improved mucosal healing in IFX treated mice. At macroscopic analysis, IFX administration leaded to a lower number of tumor lesions compared with controls, confirmed at microscopic analysis, although differences were not statistically significant. *In vitro*, IFX did not modify the proliferation ability of a murine colon tumor cell line, confirming the *in vivo* data. Taken together, our data suggest that beside its well-known healing capacity, IFX does not increase proliferative cancer cells ability and CRC risk in AOM-DSS model of tumor on chronic colitis.

MATERIALS AND METHODS

Experimental chronic colitis associated to the development of colon cancer

All experiments were approved by the Local Ethics Committee for Animal Research Studies at the Catholic University of Rome (protocol number F42/2009). The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation.

The experimental chronic colitis associated to the development of CRC was obtained in female mice C57BL/6. The animals were treated with an intraperitoneal (i.p.) injection of 10 mg/kg of Azoxymethane (AOM, Sigma-Aldrich, Munich, Germany). After one week (time 0) mice were exposed to three cycles of one week with Dextran Sodium Sulphate 2% (DSS, Molecular Weight 36-44 kDa, MP Biomedical Aurora, OH, United States) in tap water, separated by 2 wk of recovery as already shown[17,18]. Animals were divided into two groups. At the third day of each cycle of DSS the first group (IFX) was treated with IFX 5 mg/kg given intravenously in 200 μL of saline, instead the control group received a saline solution infusion. Animals were evaluated every day, by measuring weight, fecal consistence and fecal occult blood, as suggested[19,20]. Disease Activity Index (DAI) was calculated as reported[19,20]. Mice were sacrificed after three weeks from last DSS cycle. Each experiment was performed using a total number of 10 mice.

Macroscopical and microscopical tissue evaluation

At sacrifice, ulcerated and polypoid tumors were counted, photographed and marked for further analysis along with the histologic evaluation. Tumor incidence was expressed as percentage. Colon and final ileum were then fixed in formalin and then embedded in paraffin.

Intestinal inflammation and proliferative alterations, such as hyperplasia, aberrant crypt foci (ACF), gastrointestinal intraepithelial neoplasia (GIN), low grade dysplasia adenoma LGA, high grade dysplasia adenoma (HGA) and adenocarcinoma were evaluated by a single trained gastrointestinal pathologist in a blinded fashion, using a validated semiquantitative scoring system as described[18,19,21].

MTT test and evaluation of IFX on colonic cancer cell line

In order to examine the direct effects of IFX on intestinal epithelial cells proliferation and vitality, the 3-(4,5-Dimethylthiazol-2Y)-2,5Diphenyltetrazolium Bromide (MTT) test was performed on CT26 (ATCC, Teddington, United Kingdom), a murine colon cancer intestinal epithelial cell line, according to manufacturer's instructions. Briefly, MTT, a salt of tetrazolium, is transformed to insoluble crystals by the succinate-tetrazolium reductase, active only in vital cells. Crystals amount is directly proportional to the number of metabolically active cells and was measured by spectrometry at 540 nm wave length (reference wave length = 630 nm). Cells were incubated with TNF-α (25 ng/mL) or IFX (100 μg/mL) or TNF-α (25 ng/mL) plus IFX (100 μg/mL), and cell vitality was evaluated after 6, 24 and 48 h. The same setting was used after pre-incubation with TNF-α (25 ng/mL) for 24 h, followed by two washings in saline. Data are expressed as percentage *vs* control. Each analysis was repeated three times.

RESULTS

IFX ameliorates chronic colitis

We first explore the impact of IFX on clinical and histological activity in chronic colitis. IFX treated animals showed a significantly lower clinical activity index, expressed by DAI score (Figure 1A) and body weight loss when compared with controls, especially in the 18th, 44th and 54th d ($P < 0.05$) (Figure 1B). At the sacrifice, we did not observe statistically significant differences in colon length (Figure 1C). The histological examination reported a reduction of the Rachmilewitz

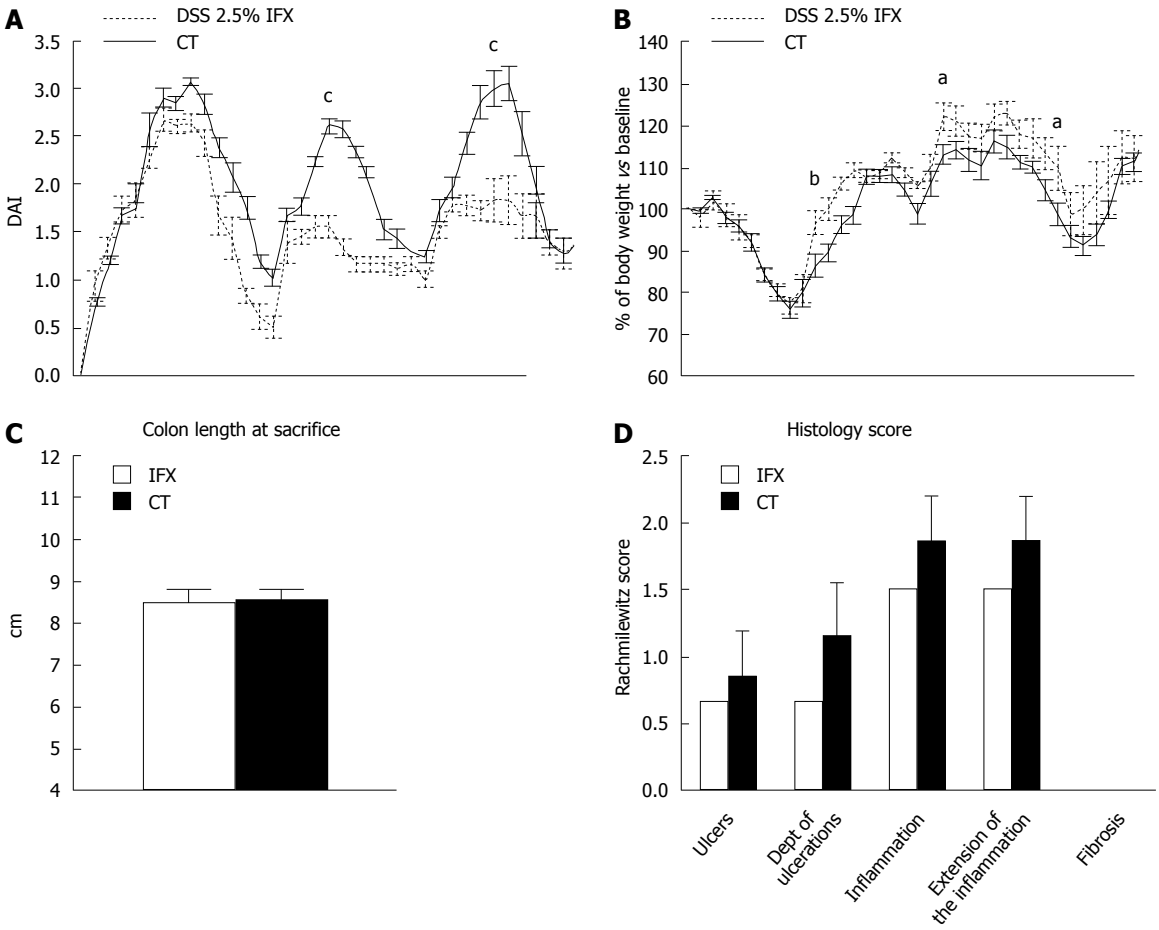

Figure 1 Effect of Infliximab on dextran sodium sulphate induced chronic colitis in mice. A: Disease activity index (DAI) in infliximab (IFX) treated animals compared with controls (CT); B: Body weight variation in IFX treated animals compared with CT; C: Colon length at sacrifice in IFX treated mice compared with CT; D: Histology assessment in IFX treated mice compared with CT. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* controls.

score in treated animals compared with controls, with a reduction in ulcer number and depth, inflammation grade and extension. However, this difference was not statistically significant. Fibrosis was absent in both groups (Figure 1D).

IFX does not increase tumorigenic risk

At sacrifice, the presence of neoplastic lesions was verified and evaluated in each animal. All animals showed at least 1 macroscopic tumor. Treated animals showed a lower number of tumors in comparison with controls, with an average of 4,5 tumors per each mouse compared with 4,4 tumors in the control group. The difference was not statistically significant ($P > 0.05$) (Figure 2A). At microscopic analysis, animals treated with IFX showed a slightly lower number of adenocarcinoma (K) with an average of 1,4 tumors per treated mouse compared with 1, 16 tumors per animal in the control group. Furthermore, in treated mice we found a non-significant higher prevalence of high grade dysplasia adenoma (HGA) compared with controls. All differences were not statistically significant ($P > 0.05$) (Figure 2B).

IFX does not affect intestinal cancer cell proliferation

In order to confirm these data, we performed an *in vitro* MTT assay, indirect expression of IFX effect on cancer cell proliferation. IFX did not significantly modify the metabolic activity of CT26 cells either directly or in association with TNF-α. On the contrary, TNF-α increased the proliferation of the neoplastic cells during the first 6 h of stimulation (Figure 3A). Pre-stimulation with TNF-α did not result in any significant variation from the first MTT assay setting (Figure 3B).

DISCUSSION

We previously showed that IFX is able to exert a local effect on intestinal mucosa[22,23]. At the same time, its potential role on the progression of colonic cancer associated to chronic colitis is crucially relevant for IBD. Our previous studies suggested that IFX is able to reduce leukocyte infiltrate, inflammatory cytokine, and adaptive and innate immunity chemokine levels, together with a reparation of the intestinal epithelial layer at scratch assay[23]. We thus expected an IFX effect on the modulation of colonic tumor progression.

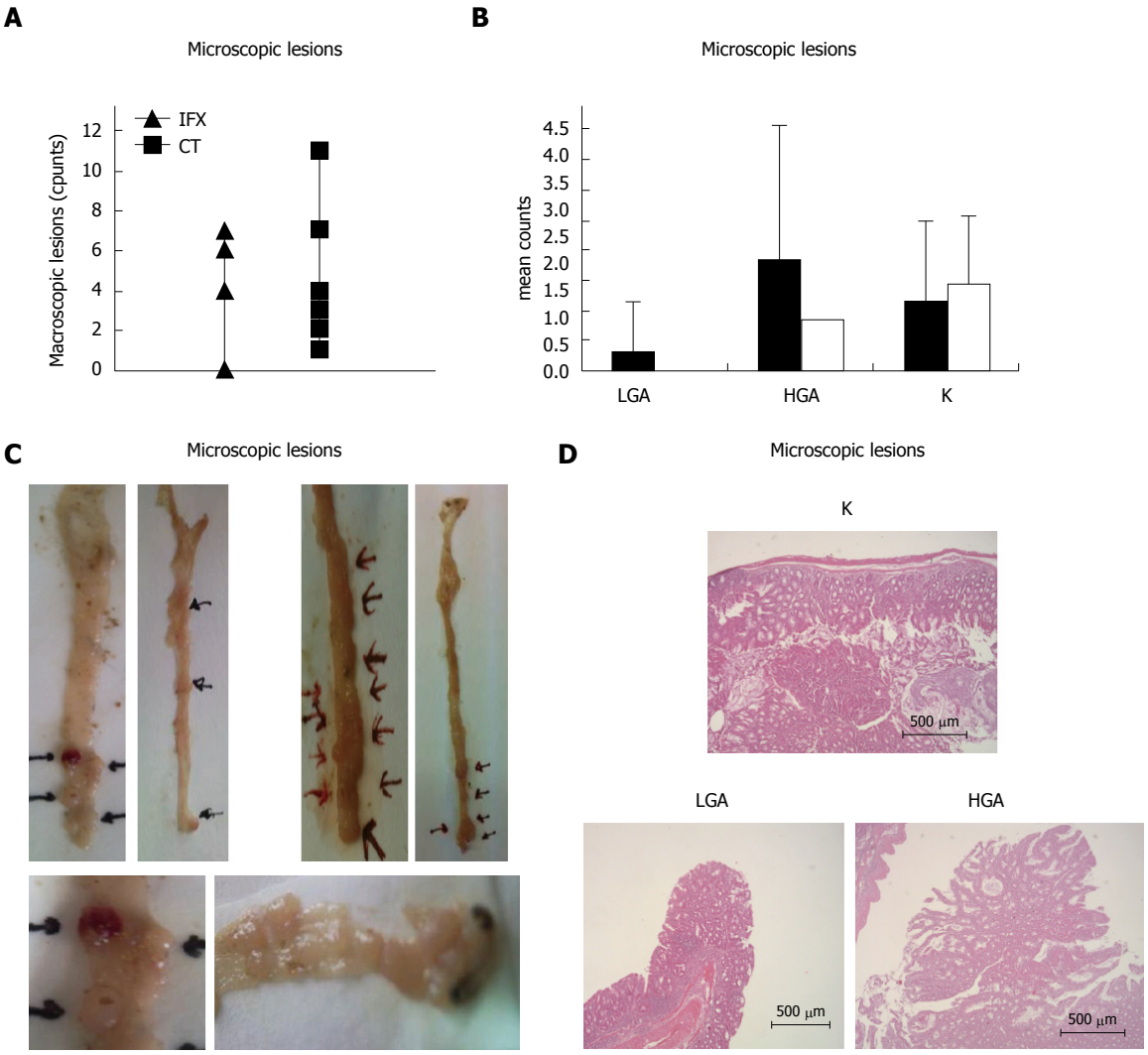

Figure 2 Effect of Infliximab on colonic cancer lesions associated to chronic colitis in mice. A: Macroscopic lesion number in Infliximab treated mice compared with controls; B: Microscopic colon lesions classified as carcinoma (K), high grade-dysplasia adenoma (HGA) and low grade-dysplasia adenoma (LGA); C: Macroscopic colon lesions (Infliximab treated on the left and controls on the right); D: Histological photos of carcinoma (K), high grade-dysplasia adenoma (HGA) and low grade-dysplasia adenoma (LGA).

Many murine models of sporadic and inflammation-related colon carcinogenesis have been developed in the last decade, including chemically induced colon cancer models, genetically engineered murine models, and xenotransplants. Among these, AOM/DSS model has proven to dramatically shorten the latency time for induction of CRC and to rapidly recapitulate the aberrant crypt foci-adenoma-carcinoma sequence that occurs in human CRC. Because of its high reproducibility, as well as the simple and affordable mode of application, the AOM/DSS has become an outstanding model for studying colon carcinogenesis. That is the main reason for choosing this model, instead of that reported by Kim *et al*[24].

In the present study, we firstly confirmed the IFX ability to reduce the clinical and histological activity of chronic colitis. This was reflected by a reduced DAI and body weight loss, as well as histological score in treated mice. Interestingly, all animals exposed to AOM and DSS expressed macroscopic tumors as expected. IFX treated animals showed a lower number of tumors compared with controls, although this difference was not statistically significant. For this reason the overall message of our experimental setting does not differ significantly from what was previously proposed in a different model[24]. Of note, microscopic analysis showed that treatment with IFX is associated to an increased prevalence of pre-neoplastic lesions (LGA and HGA) compared with controls, together with a slightly and not significant decrease of cancer lesions. This suggests that the control of chronic inflammation provided by IFX can be able to move cancer progression to a pre-cancer condition. This finding is somehow comforting if taken together with new evidences suggesting that colonic cancer risk in IBD and in particular in ulcerative colitis (UC) is lowering in recent years[25-29]. Moreover, since IBD-associated CRC can affects patients at a younger age than sporadic

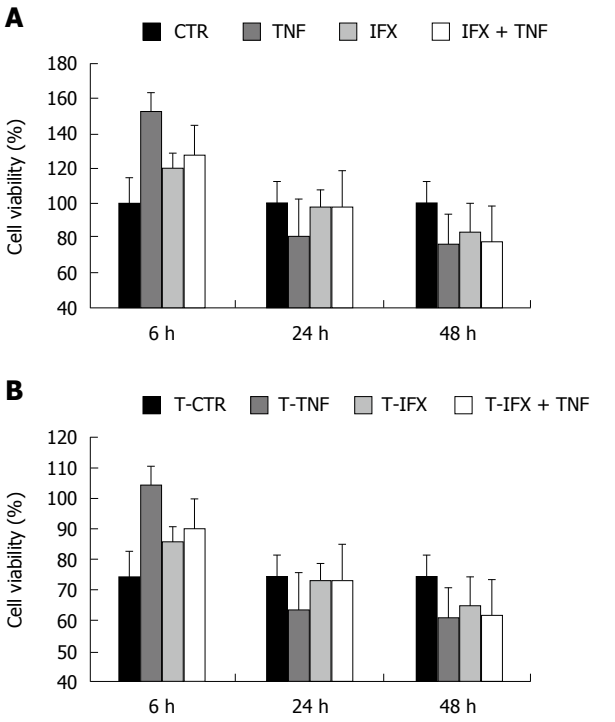

Figure 3 Effect of Infliximab on proliferation and vitality of colonic cancer cell line CT26: MTT assay. A: MTT performed without cell pre-treatment; B: MTT performed following 24 h cell pre-incubation with TNFα.

CRC[8,25,30], the finding that IFX do not dramatically move the incidence of colonic cancer in experimental colitis, makes the use of this compound in younger age even less problematic. Nonetheless, murine cancer model cannot be perfectly assimilated to what really happens in humans, since other mechanisms of action could occur. This represents a major limitation for our study. However, *in vitro* MTT assay supported the idea that IFX has not a potential proliferative role on cancer cells, since it does not increase their metabolic activity.

Overall, our study sustains a safe action of TNF-α block in chronic colitis without increasing the associated tumorigenic risk.

COMMENTS

Background

Colorectal cancer (CRC) is an important worldwide medical problem as it is the third most commonly diagnosed serious form of cancer in men and in women and represents the third leading cause of cancer death for men and for women and the second leading cause of cancer mortality overall.

Research frontiers

The authors showed that influence of infliximab (IFX) significantly reduced disease activity index and body weight loss, preserving colon length at sacrifice. Histological analysis revealed an improved mucosal healing in IFX treated mice. At macroscopic analysis, IFX administration leaded to a lower number of tumor lesions compared with controls, confirmed at microscopic analysis, although differences were not statistically significant. *In vitro*, IFX did not modify the proliferation ability of a murine colon tumor cell line, confirming the *in vivo* data.

Innovations and breakthroughs

Taken together, these data suggest that beside its well-known healing capacity, IFX does not increase proliferative cancer cells ability and CRC risk in AOM-DSS model of tumor on chronic colitis.

Applications

This study sustains a safe action of TNF-α block in chronic colitis without increasing the associated tumorigenic risk.

Peer-review

They show that IFX does not affect the development of murine colitis-associated cancer and proliferation of intestinal epithelial cells. Based on these results, the authors conclude that IFX does not increase colonic cancer risk in colitis-associated cancer. This study suggests an important message that anti-TNF-α therapy may not affect the increase cancer risk in inflammatory bowel disease patients.

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ORIGINAL ARTICLE

Basic Study

Ex vivo response to mucosal bacteria and muramyl dipeptide in inflammatory bowel disease

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Author contributions: Loganes C, Valencic E and Tommasini A designed the experimental plan and conceived and designed the research; Pin A, Marini E, De Leo L performed all experiments; Loganes C, Monasta L performed the statistical analyses; Martelossi S, Naviglio S and Not T enrolled the patients and provided patient samples; Loganes C, Tommasini A, Marcuzzi A wrote the paper; Martelossi S and Marcuzzi A revised the manuscript critically for important intellectual content; all authors read and approved the final version of this manuscript.

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Abstract

AIM

To evaluate how mucosal bacteria impact on the spontaneous and muramyl dipeptide (MDP)-induced inflammation in Crohn's disease (CD) and ulcerative colitis (UC).

METHODS

Colonic mucosal biopsies were collected from children with active or remissive CD, UC and controls. Two tissue samples were taken from inflamed mucosal segments (in patients with active disease) or from non-inflamed mucosa [in patients in remission or in healthy controls (HC)]. Experiments were performed in the presence or absence of antibiotics, to assess whether the disease-associated microbiota can modulate the cytokine response *ex vivo*. For this purpose, each specimen was half-cut to compare spontaneous and MDP-induced inflammation in the presence of live bacteria (LB) or antibiotics. After 24 h of culture, an array of 17 cytokines was assessed in supernatants. Statistical analyses were performed to find significant

differences in single cytokines or in patterns of cytokine response in the different groups.

RESULTS

We demonstrated that subjects with CD display a spontaneous production of inflammatory cytokines including granulocyte-colony stimulating factor (G-CSF), interleukin (IL) 6, IL8, IL10 and IL12, that was not significantly influenced by the addition of antibiotics. UC specimens also displayed a trend of increased spontaneous secretion of several cytokines, which however was not significant due to broader variability among patients. After the addition of antibiotics, spontaneous IL8 secretion was significantly higher in UC than in controls. In HC, a trend towards the weakening of spontaneous IL8 production was observed in the presence of live mucosal bacteria with respect to the presence of antibiotics. In contrast, in the presence of LB UC showed an increasing trend of spontaneous IL8 production, while MDP stimulation resulted in lower IL8 production in the presence of antibiotics. We also showed that subjects with CD seem to have a lowered production of IL8 in response to MDP in the presence of LB. Only with the addition of antibiotics, likely reducing the contribution of LB, multivariate statistical analysis could identify the combination of measures of G-CSF, tumor necrosis factor alpha, IL4 and IL17 as a good discriminator between CD and UC.

CONCLUSION

We showed that the presence of LB or antibiotics can significantly influence the inflammatory response *ex vivo* in inflammatory bowel diseases.

Key words: Gut-microbiota; Crohn's disease; Ulcerative colitis; Cytokines; Inflammation

Core tip: Even though previous studies have already considered cytokine secretions as marker of an inflammatory condition and mucosal imbalance in Crohn's disease (CD) subjects, they did not take into account the autochthonous colonization of the intestinal mucosa by the disease-associated microbiota. In this work we investigated whether the microbiota can modulate the *ex vivo* cytokine response, in the presence or absence of antibiotics, or if a selection of cytokines could discriminate different inflammatory bowel diseases types. Through a multivariate logistic model we identified, only in specimens treated with antibiotics, a specific cytokine profile able to discriminate CD from ulcerative colitis.

Loganes C, Valencic E, Pin A, Marini E, Martelossi S, Naviglio S, De Leo L, Not T, Monasta L, Tommasini A, Marcuzzi A. *Ex vivo* response to mucosal bacteria and muramyl dipeptide in inflammatory bowel disease. *World J Gastroenterol* 2016; 22(44): 9734-9743 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9734.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9734

INTRODUCTION

Inflammatory bowel diseases (IBD) are intestinal disorders characterized by a complex inflammatory process that may involve any part of the digestive tract. IBD include mainly Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by a different spectrum of clinical and pathological features[1,2].

In CD, the inflammation is extended through the whole intestinal wall of the gut with a segmental distribution, while in UC the inflammatory process is continuous and predominantly confined to the colonic mucosa[2].

IBD are characterized by an aberrant activation of the mucosal immune system with reaction to luminal content in genetically susceptible subjects[3], resulting in a chronic inflammatory state with dysregulation of several cytokines[4-6].

Cytokines are important mediators in the gut-associated immune system, both in physiological conditions and in disease[7]. In normal conditions, the mucosal immune activation reflects an active balance between tolerance of luminal content and response to commensal and pathogenic bacteria. Indeed, this basal activation is associated with a predominance of cytokines such as interleukin (IL) 10 and transforming growth factor beta and the production of antimicrobial peptides that can contribute to the shaping of the "healthy" microbiota. In pathological conditions, other cytokines and mediators are produced, which can, at the same time, lead to tissue injury and altered regulation of the microbiota and dysbiosis[4,8-10].

Chronic tissue damage results in clinical intestinal symptoms in IBD patients (abdominal cramping and pain, diarrhea, constipation and bowel obstruction, nausea and vomiting), even though extra-intestinal manifestations (weight loss, fever, asthenia and arthralgias) and immune disorders are also associated with this pathologies[11].

Despite the large number of studies and clinical analyses, several issues remain to be solved regarding the immune pathogenesis of IBD. In particular, the therapy of IBD is still based mainly on anti-inflammatory and immunosuppressive therapies, while mounting evidence supports the idea that defective immunity may underlie IBD pathogenesis, especially in cases with an early onset in childhood[12]. In fact, excessive inflammation in IBD could either result from a hyper-response of the immune system or it may just represent a compensatory response to luminal contest in the background of defective immunity[13].

Indeed, CD has been alternatively classified as an autoinflammatory disorder or a disease with primary immune deficiency[14].

On the one hand, some studies suggested that CD is an autoinflammatory disease, characterized by an increase in the inflammatory response due to nucleotide-binding oligomerization domain-containing protein 2 (NOD2) variants, even though data supporting this hypothesis are controversial. NOD2 is an intracellular receptor involved in gastrointestinal immunity; it detects bacterial components and has been identified as the first susceptibility gene for CD[15-17]. The binding of muramyl dipeptide (MDP) to NOD2 leads to the downstream activation of mitogen-activated protein kinases and nuclear factor-kappa beta (NF-κB) signaling, which in turn stimulates the transcription of several immune response genes. However, early studies have shown the association of the most common mutation 3020*ins*C with a reduced activation of NF-κB[18]. In contrast, later investigations proposed that the pro-inflammatory action is caused by a dysregulated autophagy[19].

On the other hand, several studies have found evidence that CD patients present immune response defects. In fact, a decreased bacterial clearance by the phagocytic system has been observed in peripheral blood cells from patients with CD; furthermore, NOD2-/- mice exhibit a reduced production of antimicrobial peptides by Paneth cells that leads to an imbalance in the microbiota in the presence of certain microbial pathogens, and intestinal inflammation can be prevented in these models by the forced expression of defensins in the intestinal cells[20].

For these reasons, we wanted to describe what happens in the disease itself, not by using simple animal models, but by studying the relationships between the mucosal immune system, influenced by its multigenic background, and the intestinal microbiota colonizing the diseased epithelium.

In these conditions, we tried to understand how the inflammatory response to stimuli such as MDP is influenced *ex vivo* by the presence of live or antibiotic-inactivated bacteria.

We performed an *ex vivo* culture of colonic explants to investigate the mucosal response both in spontaneous conditions and after the stimulation with MDP.

MATERIALS AND METHODS

Patients and gut biopsies collection

Forty-eight children with IBD and eight control subjects were enrolled. Participation in the study was proposed to all patients with IBD or their guardians when the performance of a colonoscopy was needed for clinical assessment, required for the diagnosis or the follow-up of their intestinal disease.

Patients with IBD were divided into four groups based on the type of intestinal disorder and on endoscopic mucosal disease activity: active Crohn's disease (CD, n = 23), remissive Crohn's disease (rCD, n = 3), active ulcerative colitis (UC, n = 15) and remissive ulcerative colitis (rUC, n = 7). Disease activity scores, namely the Pediatric Crohn's Disease Activity Index and the Pediatric Ulcerative Colitis Activity Index were also calculated. Any other recent acute disease or chronic inflammatory conditions are reported in these patients.

Control subjects were selected among children who underwent colonoscopy as part of their clinical investigations for various reasons not related to inflammatory enteropathies, such as routine evaluation of juvenile intestinal polyps, autoimmune gastritis, unexplained abdominal symptoms and weight loss. They had evidence of normal, non-inflamed mucosa and were thus considered healthy controls (HC).

Colonic mucosal biopsies were taken during colonoscopy. For each subject, two specimens were collected from the same mucosal area, in addition to the biopsies obtained for clinical purposes. In patients with active disease, two inflamed biopsies were collected, while in patients with inactive disease or HC two biopsies were taken from non-inflamed areas.

All subjects were recruited from the Gastroenterology and Clinical Nutrition Unit, Institute for Maternal and Child Health - IRCCS "Burlo Garofolo", Trieste, Italy and written informed consent was obtained from patients and donors (or their guardians) according to a protocol approved by the Independent Ethical Board at the Institute (n.185/08, 19/08/2008).

Ex vivo specimen culture

Immediately after collection, biopsies were cut in half following the intestinal layers' orientation and split in two tubes containing a physiological saline solution. Once they reached the laboratory, the specimens were transferred to sterile tubes with culture medium (X-VIVO, Lonza, Verviers, Belgium) supplemented with 10% human AB serum (Sigma-Aldrich, Milano, Italy) and 2 mmol/L L-glutamine (EuroClone, Milano, Italy). The intestinal tissue was exposed to 250 ng/mL MDP (N-Acetylmuramyl-L-alanyl-D-isoglutamine hydrate, Sigma-Aldrich) or medium alone, in the presence or absence of antibiotics: 100 U/mL penicillin (EuroClone) and 0.1 mg/mL streptomycin (EuroClone).

After 24 h culture at 37 ℃ in a humidified atmosphere (95% air and 5% CO_2), supernatants were collected and stored at -20 ℃ for cytokine analysis.

Cytokine quantifications

The cytokines and the chemokines released in the culture medium were measured using the Bio-Plex Pro® Human Cytokine 17-plex Assay (BioRad, Hemel Hempstead, United Kingdom), according to the manufacturer's instructions.

Analytes examined by this method were IL1β, IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12 (p70), IL13, IL17, granulocyte-colony stimulating factor (G-CSF), GM-CSF, IFNγ, MCP1 (MCAF), MIP1β, and TNFα.

Samples were analyzed with the Bio-Plex® 200 reader and data were managed using Bio-Plex Manager®

Table 1 Characteristics of patients included in the study

Patients (n = 56)		Age (yr) (mean ± SD)	Male/female	Active/ remission
IBD (n = 48)	CD (n = 26)	11 ± 3.08	16/10	23/3
	UC (n = 22)	15 ± 3.88	12/10	15/7
CONTROLS (n = 8)	HC (n = 8)	10 ± 4.60	4/4	-

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; HC: Healthy controls.

Table 2 Multivariate logistic model for inflammatory bowel disease patients *vs* healthy controls in the presence of antibiotics and without muramyl dipeptide stimulation

Variables	OR	95%CI	*P* value	Regression coefficients
IL2	1.553421	1.011162-2.386481	0.044	0.440460
IL4	0.473238	0.228780-0.978906	0.044	-0.748157
IL8	1.000267	1.000030-1.000504	0.027	0.000267
Constant	0.065259	0.003533-1.205600	0.067	-2.729393

software, which returned data as Median Fluorescence Intensity and concentration (pg/mL).

Statistical analysis

All statistical analyses were carried out using Stata/IC 14.1 (StataCorp LP, College Station, United States) and GraphPad Prism software version 5 (GraphPad, San Diego, United States). Values assumed by cytokines were described as medians and interquartile ranges and represented with box plots using Tukey's whiskers. Statistical significance was set as $P < 0.05$ (a), $P < 0.01$ (b) and $P < 0.001$ (c).

Associations between cytokines and specific conditions (HC *vs* IBD, and CD *vs* UC) were analyzed firstly by univariate logistic regression and then by multivariate logistic regression. For the multiple comparisons carried out by univariate logistic regression, considering the relatively small sample size, which implied having relatively large P values, we decided not to apply any correction to the P value for statistical significance: we realize that we carried out independent univariate logistic regressions on different outcomes and separately for 17 cytokines, chemokines and growth factors. However, this just is considered as a preliminary step for the selection of cytokines to be included in the multivariate analysis: significant variables ($P < 0.05$ in the univariate analysis) were entered into multivariate analysis. Multivariate logistic regression analysis was employed to determine cytokine profiles associated with the different forms of IBD. Receiver operating characteristic (ROC) curves were constructed to evaluate the performance of the multivariate models. The area under the curve (AUC), sensitivity and specificity were also calculated. For each model, we identified the optimal cut-off by maximizing the sum of sensitivity and specificity.

RESULTS

Experimental design

Clinical characteristics of all patients included in the study, obtained from the medical sheets and from the colonoscopy records, are reported in Supplementary Table 1 and Supplementary Table 2 and summarized in Table 1.

Ex vivo culture of biopsies is thought to reproduce both the immunological and the microbiologic background of the disease. However, to isolate the contribution of live bacteria (LB) to inflammation, biopsies were stimulated in absence or in presence of antibiotics. Thus, for each sample we examined four conditions: basal with LB or with antibiotics (PS), and MDP-stimulated with (PS + MDP) or without antibiotics (LB + MDP).

After a 24 h culture, the media were harvested to proceed with the quantification of 17 analytes, including cytokines, chemokines and growth factors (Figure 1).

The levels of three cytokines (IL5, IL7 and IL13) were under the lower limit of detection in all evaluated conditions and were thus excluded.

Basal cytokine network in IBD

In basal conditions with live mucosal bacteria, five cytokines had levels significantly higher in CD than in controls (HC) (CD *vs* HC: G-CSF, $P = 0.024$, IL6 $P = 0.037$, IL8 $P = 0.033$, IL10 $P = 0.049$, IL12, $P = 0.028$); levels of most cytokines tended to be even higher in UC, but because of the high variability among patients, differences did not reach significance, except for GM-CSF, whose levels were higher in UC compared with both HC ($P = 0.031$) and CD ($P = 0.034$) (Figure 2).

Levels of IL1β, IL2, IL4, IL17, IFNγ, TNFα, MCP1 and MIP1β did not differ significantly between the three studied groups, even if median levels of several cytokines such as IL2, IL4 and TNFα tended to be higher in UC than in HC. In general, HC showed a narrower variability compared to patients (Supplementary Figure 1).

Median levels of all cytokines in the different groups and conditions are rendered in Figure 3 with a color analog scale (CAS): for each cytokine, values 0-24% of the higher value are displayed in green, 25%-49% in orange, 50%-74% in red and above 75% in dark red.

In specimens from patients with endoscopically inactive disease, almost all cytokines were detected, as expected, at lower levels than in active disease. The decrease is significant in UC for GM-CSF ($P < 0.01$), IL6 ($P < 0.05$), IL10 ($P < 0.05$) and TNFα ($P < 0.05$) (Data not shown).

The addition of antibiotics in the bioptic culture to inhibit mucosal bacteria did not lead to relevant changes in the secretion pattern (Supplementary Figure 2), with the exception of IL8 in UC samples.

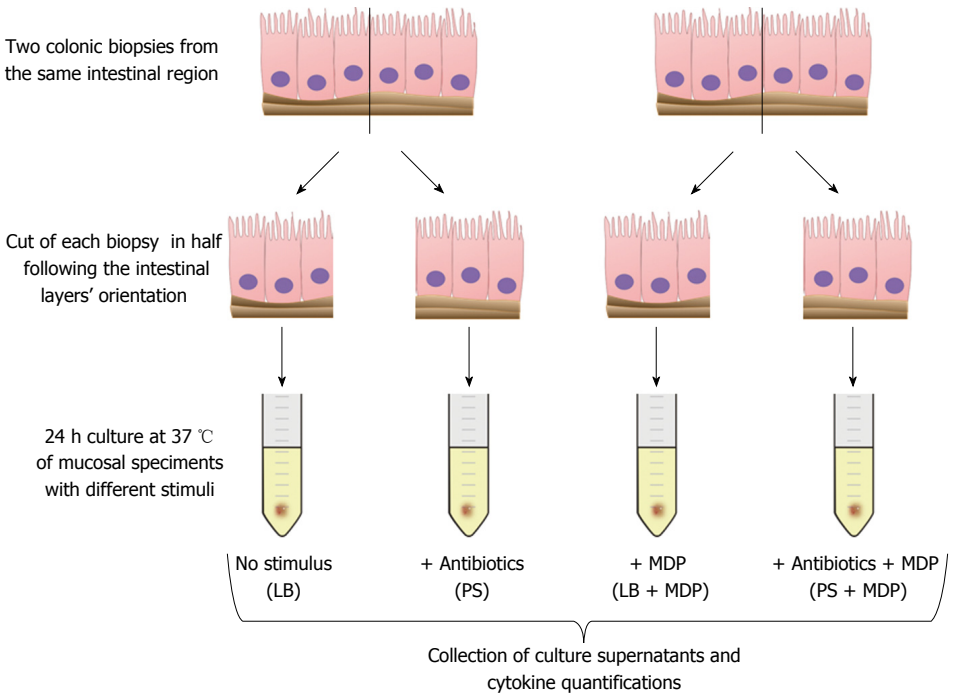

Figure 1 Schematic culture protocol of colonic biopsies derived from colonoscopy. LB: Live Bacteria; PS: Penicillin-streptomicin; MDP: Muramyl dipeptide.

Table 3 Multivariate logistic model for inflammatory bowel disease patients *vs* healthy controls in the presence of antibiotics and muramyl dipeptide stimulation

Variables	OR	95%CI	*P* value	Regression coefficients
MCP1	0.997248	0.995067-0.999434	0.014	-0.0027555
IL8	1.000094	1.000019-1.000170	0.014	0.0000944
IL10	1.098966	1.009008-1.196944	0.030	0.0943694
Constant	0.529026	0.108222-2.586054	0.432	-0.636717

Table 4 Multivariate logistic model for Crohn's disease *vs* ulcerative colitis in the presence of antibiotics and muramyl dipeptide stimulation

Variables	OR	95%CI	*P* value	Regression coefficients
G-CSF	0.999703	0.999484-0.999921	0.008	-0.0002975
TNFα	0.990492	0.981420-0.999649	0.042	-0.0095533
IL4	1.155281	1.012918-1.317651	0.031	0.1443433
IL17	1.009361	1.000308-1.018497	0.043	0.0093177
Constant	1.043247	0.333344-3.264992	0.924	0.042338

The trend of IL8 increase after antibiotics in UC is so clear that the difference compared with HC becomes significant (Figure 4).

Cytokine network induced by MDP

MDP was used to investigate the function of the NOD2-pathway *ex vivo* in CD compared with UC and HC.

In general, the response to MDP appeared to be higher in UC than in CD (see the CAS in Figure 3). After stimulation with MDP in the presence of LB, even though the differences are not significant, there is a clear trend of increased secretion of IL2, IL6, IL8, IL17 and IFNγ in UC, while no change was observed in CD. Indeed, even if in a smaller scale, HC tended to secrete reduced amounts of IL2, IL6 and IFNγ (Figure 5).

However, after the addition of antibiotics, this pattern was subverted and MDP stimulation failed to produce an evident increase in cytokine secretion in any group and it was even possible to note a reduced trend of secretion for IL8 in UC (Figure 6).

No variation was noted in the remaining cytokines, whose values are reported in supplementary Tables 3 and 4.

Multivariate logistic regression and ROC analysis to identify profiles of pro-inflammatory cytokines associated with IBD diagnosis

Multivariate logistic regression were carried out to assess if any cytokine combination was associated with IBD related outcomes.

Only experiments in the presence of antibiotics provided profiles significantly associated with IBD. In particular, without MDP stimulation, IL2, IL4 and IL8 were simultaneously associated with IBD in a multivariate analysis (Table 2). ROC analysis (Figure 7A) shows that the AUC was 0.98 (95%CI: 0.93-1.00), and we could have a sensitivity of 100% with a specificity of 71%.

After MDP stimulation another set of cytokines (MCP1, IL8 and IL10) was significantly associated with IBD (Table 3). The AUC for this model was 0.91 (95%CI: 0.79-1.00) (Figure 7B). Keeping sensitivity

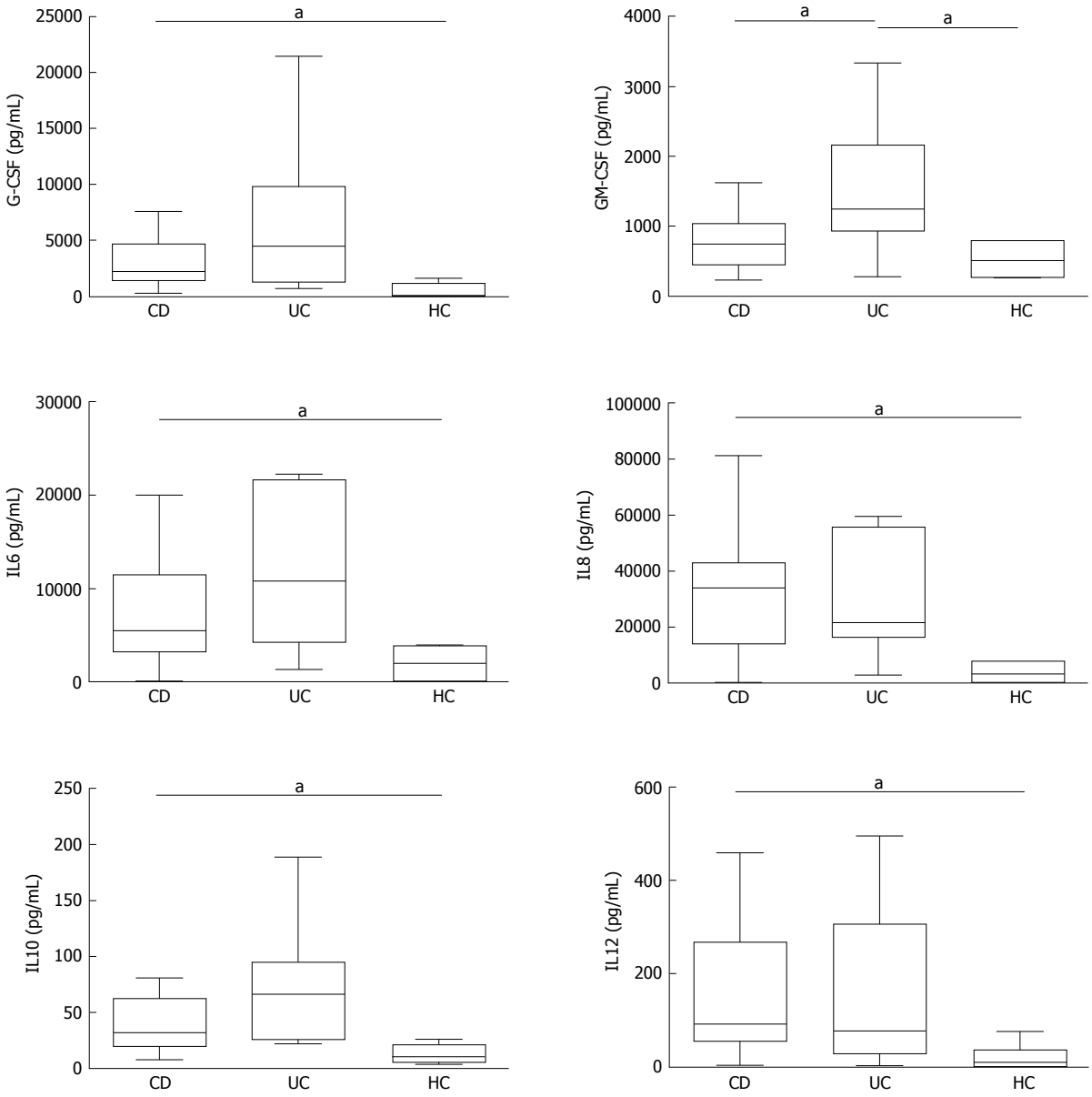

Figure 2 Cytokine secretion levels by colonic biopsies from inflammatory bowel diseases patients. Levels of cytokine secretion by colonic biopsies from inflammatory bowel diseases (IBD) patients in active phase [Crohn's disease (CD); ulcerative colitis (UC)] and healthy controls (HC) in basal condition [live bacteria (LB)]. Statistical significances are denoted using alphabetical letters ($^{a}P < 0.05$).

for the detection of IBD at 94%, we obtained a specificity of 75%, which would permit to identify 45 IBD patients out of 48, with 2 false positives out of 8 HC.

After MDP stimulation it was also possible to discriminate between CD and UC based on the measure of G-CSF, TNFα, IL4 and IL17 (Table 4). According to the ROC analysis (Figure 7C), the AUC was 0.78 (95%CI: 0.65-0.91), and we could obtain a sensitivity on CD of 92.31% with a specificity of 50%.

DISCUSSION

It is well known that subjects with active IBD display increased secretion of several inflammatory cytokines in the intestinal mucosa. For example, aberrant secretions of IL8 after challenge with *E. coli* or other intestinal bacteria were recorded by Edward and colleagues and considered as a marker of the mucosal imbalance typical of CD[21]. The authors, however, did not take into account the autochthonous colonization of the intestinal mucosa by the disease-associated microbiota. In the present work, we compared the cytokine profile of the intestinal mucosa in the presence or absence of antibiotics, to assess whether disease-associated microbiota can modulate the cytokine response *ex vivo*. We demonstrated that subjects with CD display a spontaneous production of inflammatory cytokines besides IL8, and including G-CSF, IL6, IL10 and IL12. The addition of antibiotics to the culture produced only a slight trend towards increased spontaneous cytokine secretion, both in CD and in UC, but no

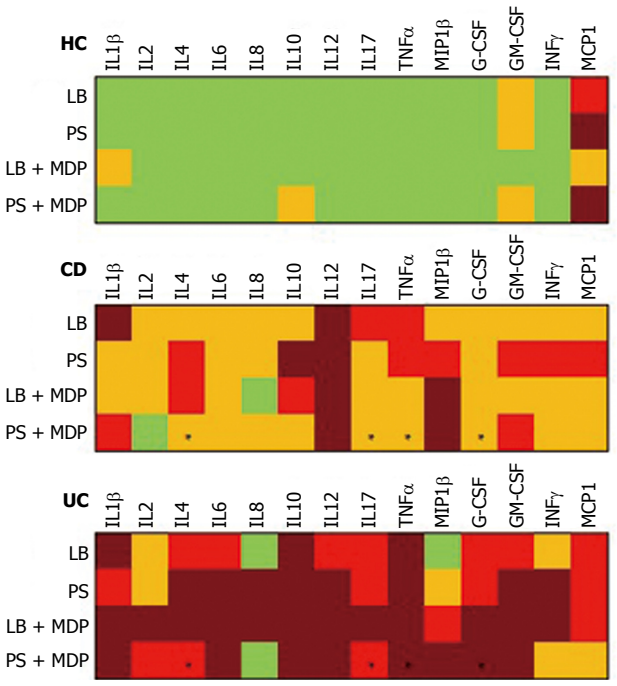

Figure 3 Color analog scale representing the median levels of all cytokines analyzed. Median levels of all cytokines in healthy controls (HC), Crohn's disease (CD) and ulcerative colitis (UC) groups in the four experimental conditions: live bacteria (LB), antibiotics (PS), live bacteria + Muramyl dipeptide (LB + MDP) and antibiotics + MDP (PS + MDP). For each cytokine, values 0-24% of the higher value are displayed in green, 25%-49% in orange, 50%-74% in red and above 75% in dark red. Asterisks indicate the set of cytokines whose measure allowed to discriminate CD from UC with 92% sensitivity and 50% specificity.

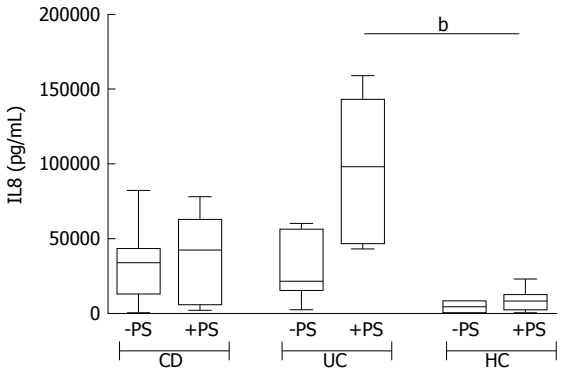

Figure 4 Dosage of IL8 secretion by colonic biopsies from inflammatory bowel diseases patients. Levels of IL8 secretion by colonic biopsies from patients with active Crohn's disease (CD), active ulcerative colitis (UC) and from healthy controls (HC), in the presence (+PS) or absence (-PS, live bacteria) of antibiotic mixture. Statistical significances are denoted using alphabetical letters ($^{b}P < 0.01$).

difference was significant, except for increased IL8 secretion in UC. These data may support the idea that antibiotics can influence UC, as suggested by other authors[22]. However, whilst some protocols that include metronidazole *in vivo* are shown to have protective effects on disease activity[22], other combinations had no effect[23]. Actually, it is well known that the risk of CD can be raised by previous use of antibiotics in

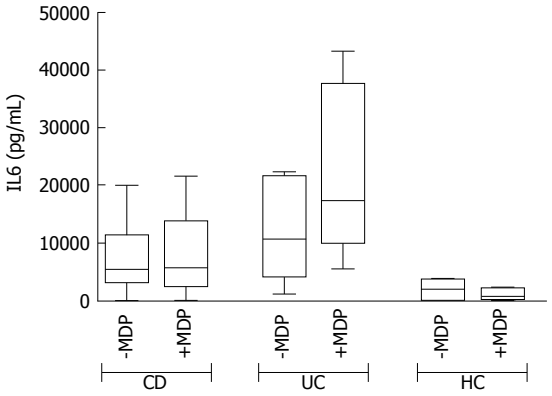

Figure 5 Representative trend of cytokine secretion after muramyl dipeptide stimulation, with live bacteria. Variation trend of IL6 secretion after muramyl dipeptide (MDP) stimulation in patients suffering from active Crohn's disease (CD), active ulcerative colitis (UC) and healthy controls (HC), in absence of antibiotics [live bacteria (LB)]. No statistical significance was found.

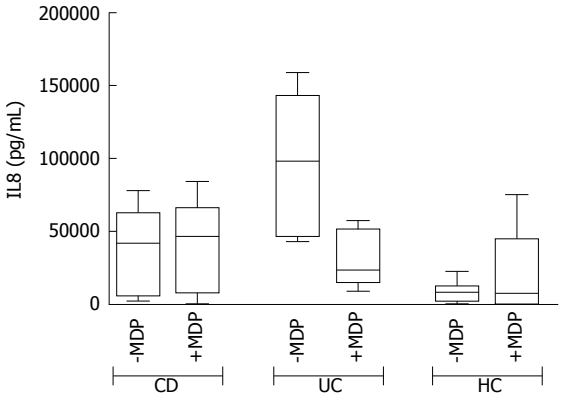

Figure 6 Representative trend of cytokine secretion after muramyl dipeptide stimulation, with antibiotics. Variation trend of IL8 secretion after muramyl dipeptide (MDP) stimulation in patients suffering from active Crohn's disease (CD), active ulcerative colitis (UC) and healthy controls (HC), in presence of antibiotics (+PS). No statistical significance was found.

childhood, but this is not believed to occur for UC[24]. Thus, the significance of increased levels of IL8 in UC after the addition of antibiotics remains without a clear explanation.

Even if the difference is not significant, there is a trend toward higher concentrations of inflammatory cytokines in UC if compared to CD. Actually, this is not surprising if we consider that we are sampling just superficial specimens where UC is expected to be more expressed than CD, which, on the contrary, may extend the inflammatory process throughout the whole intestinal wall[25].

Of note, in HC, the IL8 secretion with or without MDP is reduced in the presence of LB if compared to antibiotics. This effect is likely due to a modulatory effect of LB on mucosal immunity. In contrast, samples from UC showed a trend of increase of cytokines such as IL6 and IL8 when stimulated with MDP in the presence of LB. However, in the presence of antibiotics, stimulation with MDP was associated with decreased

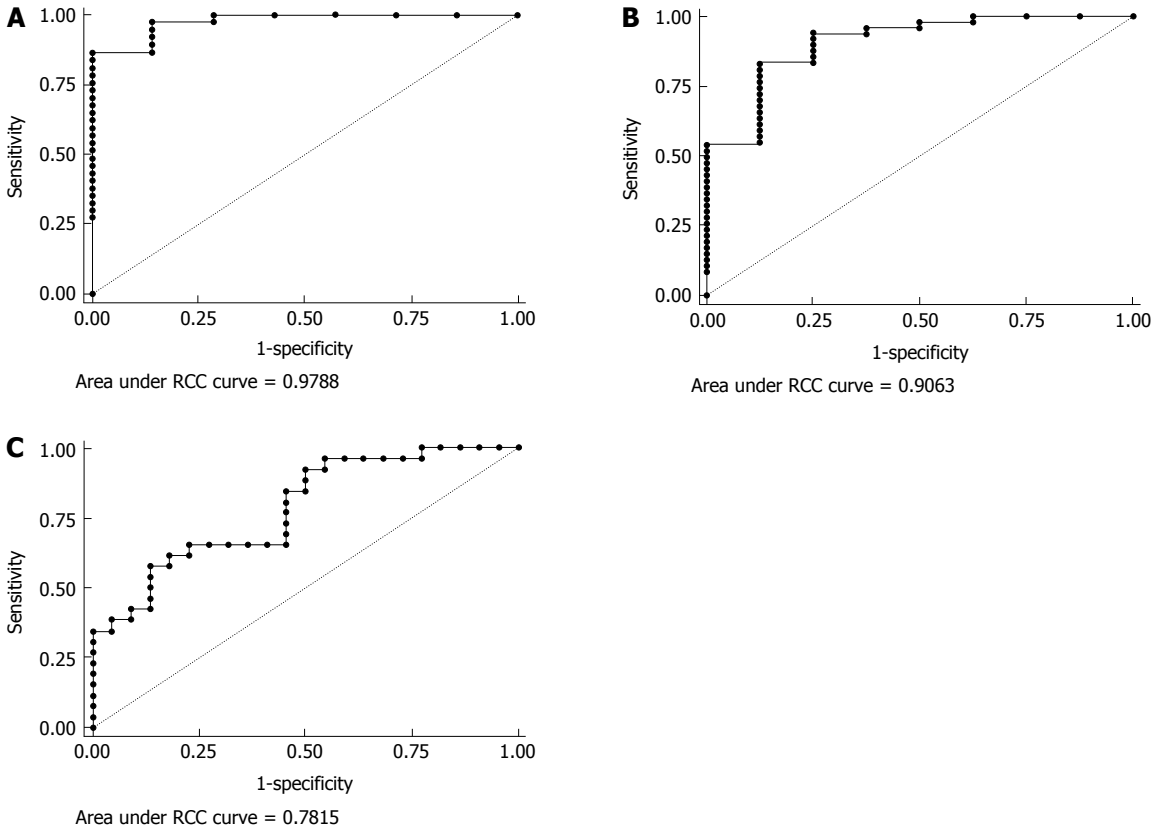

Figure 7 Multivariate combinations. A: Roc curve of IL2, IL4 and IL8: the best cutoff value was for a sensitivity of 100% and specificity of 71% to discriminate IBD (UC and CD) from healthy controls (AUC = 0.98, 95%CI: 0.93-1.00); B: Roc curve of MCP1, IL8 and IL10: the best cutoff value was for a sensitivity of 94% and specificity of 75% to discriminate IBD (UC and CD) from healthy controls (AUC = 0.91, 95%CI: 0.79-1.00); C: Roc curve of G-CSF, TNFα, IL4 and IL17: the best cutoff value was for a sensitivity of 92% and specificity of 50% to discriminate CD from UC (AUC = 0.78, 95%CI: 0.65-0.91).

IL8 secretion. Thus, antibiotics alone seem to increase IL8 production in UC, either because of a loss of the protective effect of mucosal bacteria or for the release of pro-inflammatory bacterial compounds. Conversely, we observed a reduction of IL8 production in UC when MDP is used together with antibiotics. Even though there is no clear explanation for this phenomenon, we can argue that in this case antibiotics and MDP-induced peptides may synergize in controlling both protective and harmful bacteria[26].

We also showed that subjects with CD seem to have a lowered production of IL8 in response to MDP in the presence of LB. Although it is not clear whether defects in NOD2 signaling might play a role in this finding, these results support the idea that CD is not usually associated with a hyper-inflammatory response to MDP, *i.e.,* it is not just an autoinflammatory disorder. Recent data suggest that inflammation in CD may be the result of compensatory responses to defective immunity[27].

Although it was not the primary aim of our work, we could demonstrate that culture with antibiotics is the only *ex vivo* condition in which it was possible to identify profiles of cytokine secretions able to discriminate between CD and UC with good sensitivity and specificity. Indeed, the combination of measures of G-CSF, TNFα, IL4 and IL17 was shown to identify CD with respect to UC with sensitivity and specificity respectively of 92% and 50%. Rather than being used to assist diagnosis, which relies only on clinical and histological features, these results highlight once more the importance of taking into account the presence of mucosal bacteria when dealing with *ex vivo* analyses of IBD.

Among the limitations of this study, we need to mention the small sample size. The decision not to apply corrections to the p values for significance for multiple univariate comparisons was a consequence of this constrain and it should be taken into account.

However, despite the small sample size, valid results have been obtained with this *ex vivo* mucosal culture, that allowed us to discern the two IBD forms.

In conclusion, we showed that CD and UC have distinct cytokine profiles in the intestinal mucosa and that the mucosal-associated microbiota can differentially impact on the inflammatory response in the two conditions.

COMMENTS

Background

Inflammatory bowel diseases (IBD) are associated with an unbalanced crosstalk between the mucosal immune system and the luminal content. Production of cytokines and antimicrobial-peptides in gut mucosa may contribute to the shaping of an altered intestinal microbiota and, conversely, microbes can

translocate across the epithelium and induce an excessive inflammatory response and recruit adaptive immunity. Treatment of IBD with antibiotics, however, gave inconsistent results as different bacterial species may have protective or harmful effects.

Research frontiers

Studies on the pathogenesis of IBD should involve the specific disease-associated microbiota together with the mucosal immunity, with its genetic background. Thus, *ex vivo* experiments that bring together these two factors are of particular importance to unravel the pathogenesis of IBD.

Innovations and breakthroughs

By reproducing four different conditions, we could dissect the effect of disease-associated live or antibiotic-inactivated bacteria on the mucosal response to muramyl dipeptide (MDP). We showed that live bacteria affect the response to MDP in different ways in Crohn's disease (CD) and in ulcerative colitis (UC).

Applications

Ex vivo analyses of the inflammatory response in the presence of antibiotics and soluble stimuli may allow studying the action of different antibiotics in patients with CD or UC.

Terminology

MDP represents a pro-inflammatory stimulus used to mimic the inflammatory activation.

Peer-review

Some strengths of the article include: an adequate sample to test the hypothesis, novel study looking at the autochthonous colonization of the intestinal mucosa by the disease-associated microbiota, and seemingly good methods to address the question. It also provides a novel method to identify CD and UC based off of specific cytokines markers.

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ORIGINAL ARTICLE

Basic Study

Angiopoietin-2/angiopoietin-1 as non-invasive biomarker of cirrhosis in chronic hepatitis C

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Author contributions: Hernández-Bartolomé A contributed to acquisition, analysis and interpretation of data, final approval given; López-Rodríguez R contributed to acquisition, analysis and interpretation of data, critical revision of the manuscript, final approval given; Borque MJ contributed to acquisition of data; González-Moreno L, Real-Martínez Y, García-Buey L and Moreno-Otero R contributed to clinical management of patients, critical revision of the manuscript, final approval given; Sanz-Cameno P contributed to conception and design of the study, analysis and interpretation of data, drafting of the manuscript and final approval given.

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Abstract

AIM

To evaluate the efficacy of peripheral blood concentrations of angiopoietins (Ang) as cirrhosis biomarkers of chronic hepatitis C (CHC).

METHODS

Ang1 and Ang2 serum levels were measured by enzyme-linked immunosorbent assays (ELISA) in samples from 179 cirrhotic and non-cirrhotic CHC patients, classified according to the METAVIR system.

Groups were compared by non-parametric Mann-Whitney *U* test. Subsequently, the association of peripheral concentrations of angiopoietins with the stage of fibrosis was analyzed using Spearman correlation test. Finally, the accuracy, sensitivity and specificity of circulating angiopoietins for cirrhosis diagnosis were determined by the study of the respective area under the curve of receiver operator characteristics (AUC-ROC).

RESULTS

Peripheral blood concentrations of Ang1 and Ang2 in CHC patients were significantly related to fibrosis. While Ang1 was decreased in cirrhotic subjects compared to non-cirrhotic ($P < 0.0001$), Ang2 was significantly increased as CHC progressed to the end stage of liver disease ($P < 0.0001$). Consequently, Ang2/Ang1 ratio was notably amplified and significantly correlated with fibrosis ($P < 0.0001$). Interestingly, the individual performance of each angiopoietin for the diagnosis of cirrhosis reached notable AUC-ROC values (above 0.7, both), but the Ang2/Ang1 ratio was much better (AUC-ROC = 0.810) and displayed outstanding values of sensitivity (71%), specificity (84%) and accuracy (82.1%) at the optimal cut-off (10.33). Furthermore, Ang2/Ang1 ratio improved the performance of many other previously described biomarkers or scores of liver cirrhosis in CHC.

CONCLUSION

Ang2/Ang1 ratio might constitute a useful tool for monitoring the progression of chronic liver disease towards cirrhosis and play an important role as therapeutic target.

Key words: Chronic hepatitis C; Area under the curve of receiver operator characteristics; Liver fibrosis; Cirrhosis; Angiopoietin-2; Angiopoietin-1; Biomarker; Angiogenesis

Core tip: Chronic hepatitis C (CHC) is the leading cause of cirrhosis and hepatocellular carcinoma and monitoring of liver fibrosis is essential for the prognosis and treatment of these patients. Liver biopsy, the gold standard for fibrosis determination, is invasive and costly. Therefore, novel reliable non-invasive biomarkers are crucial for CHC management. Angiogenesis is closely related to the pathogenesis of the disease and angiopoietins play a relevant role in this process. Interestingly, this study confirms the valuable association of circulating angiopoitein-1 (Ang1) and angiopoitein-2 (Ang2) levels with CHC progression and reveals the outstanding role of Ang2/Ang1 ratio as potential non-invasive biomarker of cirrhosis.

Hernández-Bartolomé A, López-Rodríguez R, Borque MJ, González-Moreno L, Real-Martínez Y, García-Buey L, Moreno-Otero R, Sanz-Cameno P. Angiopoietin-2/angiopoietin-1 as non-invasive biomarker of cirrhosis in chronic hepatitis C. *World J Gastroenterol* 2016; 22(44): 9744-9751 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9744.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9744

INTRODUCTION

Chronic liver disease (CLD) caused by hepatitis C virus (HCV) is an important public health problem worldwide. Nowadays, the number of patients with HCV-related cirrhosis is increasing and at this stage of the disease serious complications, such as bleeding esophageal varices or hepatocellular carcinoma (HCC) development, can take place[1-4]. Although the new medication based on direct-acting antivirals (DAAs) is very efficient for chronic hepatitis C (CHC) treatment, the access of numerous patients to these novel therapies is difficult because of their elevated cost. In addition, the silent course of disease often leads to many undiagnosed subjects[4-6].

An important feature of CHC progression is the persistence of HCV in the liver, which perpetuates the inflammatory response and deregulates other repairing processes, leading to angiogenesis, fibrosis, cirrhosis and HCC. Liver fibrosis is characterized by the replacement of hepatocytes by extracellular matrix (ECM), particularly collagen and several extracellular matrix proteins whose organization in non-soluble complex polymers generates the architectural and functional disorganization of the liver[7-10]. Simultaneously, chronic liver injury leads to the development of abnormal intrahepatic vasculature in a fundamental attempt to reestablish the metabolic interchange between blood and the injured tissue[11-14]. Indeed, pathological angiogenesis has been reported in diverse CLD and in the context of different inflammatory, fibrotic, and ischemic conditions as well as in HCC[13,15-18].

Among the mechanisms that closely modulate the angiogenic process, the Angiopoietins/Tie2 system is considered to play a pivotal role during the late phase of angiogenesis and is responsible for the maturation of newly formed vascular structures[19-21]. The correct regulation of the tyrosine kinase Tie2 is essential for normal vascular development[22,23]. Angiopoitein-1 (Ang1) and angiopoitein-2 (Ang2) have similar affinity toward Tie2 but their effects are quite different and context dependent[24-26]. Interestingly, the balance between both angiopoietins is altered in several CLD diseases, with its highest manifestation in HCC[13,27].

The knowledge of the fibrosis stage and progression rate is crucial for prognosis and treatment of CHC patients[28], but it is quite difficult to achieve since liver biopsy, the unique clinically accepted tool to evaluate the advance of the disease, has many drawbacks such as its invasiveness and elevated cost[29-31]. Therefore, alternative strategies are being actively investigated in

order to reduce or avoid the need of liver biopsies for the assessment of liver disease[32,33].

In this regard, the close relationship between liver fibrosis and pathological angiogenesis, together with the observed imbalance of angiopoietins levels in different CLD, pointed us to evaluate the usefulness of these angiogenic factors as non-invasive biomarkers of CHC progression[34,35]. Therefore, this study was designed to assess the levels of Ang-1 and Ang-2 in the serum of CHC patients with or without cirrhosis and to estimate their potential diagnostic value.

MATERIALS AND METHODS

Patients

The study included 179 serum samples from CHC patients without human immunodeficiency virus (HIV), hepatitis B or other liver diseases who had undergone liver biopsy for clinical purposes and gave written informed consent for their experimental use.

The study protocol was approved by the Clinical Research Ethics Committee of Hospital Universitario de La Princesa and adhered to the rules of the Declaration of Helsinki. The diagnosis of CHC was confirmed by the presence of serum HCV-RNA assayed with the reverse transcription polymerase chain reaction (RT-PCR) method (Amplicor Roche Molecular System, Branchburg, NJ). The genotype of HCV was determined by reverse-hybridization line probe assay (INNO-LiPAHCV; Innogenetics, Zwijndreht, Belgium). Also, immediately prior to liver biopsy, a blood sample was taken from each patient to analyse routine biochemical and clinical parameters using standard methods.

Liver histology

Liver biopsy tissue was obtained from all patients by percutaneous needle extraction (HepafixH, B. Braun Melsungen AG, Melsungen, Germany) under ecographic control. All liver biopsy specimens were fixed in 5% buffered formalin and embedded in paraffin for routine anatomo-pathological examination. Liver fibrosis was staged as F0 to F4 according to the METAVIR classification system[36]. In order to simplify, 3 patients with F0 were included in the F1 group.

Determination of serum Ang1 and Ang2 levels

Concentrations of Ang1 and Ang2 were measured in serum samples from all patients with CHC obtained on the same day that they had undergone percutaneous liver biopsy. According to the manufacturer's directions, levels of Ang1 and Ang2 in serum were evaluated using human enzyme-linked immunosorbent assay (ELISA) Kits (Quantikine: R&D Systems, Minneapolis, MN). Once the reaction was stopped, the absorbance of each well was determined using a microplate reader (Bio-Rad). Concentrations of Ang1 and Ang2 were obtained from the standard curve. All assays were done by duplicate and the mean concentration was calculated.

Statistical analysis

All data were analyzed with SPSS version 16.0 software (SPSS Inc., Chicago, IL, United States) and expressed as median values or in percentages, except for age (median and range). Comparisons of Ang1 and Ang2 serum levels between groups of cirrhotic and non-cirrhotic patients were performed by non-parametric Mann-Whitney *U* test. The association of angiopoietins with liver fibrosis was analyzed by the Spearman correlation test. Two-tailed *P* values below 0.05 were considered statistically significant. Receiver operating characteristics (ROC) curves were applied to evaluate the diagnostic precision of angiopoietins and their ratio (Ang2/Ang1) to identify CHC patients with cirrhosis. In addition, different parameters of clinical relevance, such as sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratio (LR), and accuracy (ACC), at different cut-off values (Youden Index - which corresponds to the maximum sum of sensitivity and specificity, 90% sensitivity or 90% specificity) were also determined. Statistic differences among different cirrhosis indices were calculated by De Long test (version 12.3.0.0, MedCalc, MariaKerke, Belgium).

RESULTS

Patient characteristics

Demographic and clinical characteristics of CHC patients are shown in Table 1. Compared with non-cirrhotic, patients with cirrhosis had lower levels of platelets and albumin but higher levels of bilirubin and transaminases [except alkaline phosphatase (ALP)]. No significant differences were found with regard to cholesterol, age, viral load or gender.

Angiopoietins levels in serum of CHC patients

In order to measure serum concentrations of Ang1 and Ang2 in the peripheral blood of CHC patients ELISA assays were performed. Interestingly, the concentration of Ang1 decreased progressively in relation to the stage of liver fibrosis whereas Ang2 levels showed the opposite tendency (Figure 1). Furthermore, the concentration of Ang1 in the serum of cirrhotic patients was significantly lower when compared to the non-cirrhotic groups ($P < 0.001$); On the contrary, Ang2 serum levels were considerably higher in patients with cirrhosis ($P < 0.01$, Figure 1). Hence, differences among fibrosis stages were more evident for Ang2/Ang1 ratio, which was further able to significantly discriminate $F > 1$.

Consequently, Spearman correlation revealed an important association of circulating levels of angiopoietins with fibrosis stage, in accordance to the results shown above: while Ang1 levels were inversely related, Ang2 and Ang2/Ang1 ratio were directly associated ($P < 0.0001$, all) as Table 2 shows.

Table 1 Baseline characteristics of chronic hepatitis C patients

Patients	Cirrhotic (n = 31)	Non-cirrhotic (n = 148)	P value
Sex (M/F)	23/8	92/56	0.20
Age (yr)	48 (24-63)	44 (22-67)	0.23
Viral load (× 10^5 IU/mL)	5.0 (3.5-11.0)	7.1 (1.8-13.0)	0.68
HCV genotype, n (%)			-
1	27 (87.1)	117 (79.1)	
Non-1	4 (12.9)	31 (20.9)	
Stage of liver fibrosis, n (%)			-
F1	-	42 (28.4)	
F2	-	66 (44.6)	
F3	-	40 (27.0)	
F4	31 (100.0)	-	
AST (UI/L)	99.0 (60.0-123.0)	48.0 (33.0-65.0)	< 0.001
ALT (UI/L)	116.0 (87.0-161.0)	74.0 (55.3-111.5)	< 0.001
ALP (UI/L)	116.0 (75.0-220.0)	126.5 (77.5-164.8)	0.39
GGT (UI/L)	91.0 (68.0-172.0)	36.0 (23.0-65.8)	< 0.001
INR	1.1 (1.1-1.2)	1.0 (0.9-1.1)	< 0.001
Bilirubin (mg/dL)	0.9 (0.7-1.1)	0.6 (0.5-0.8)	< 0.001
Platelet count (× 10^9/L)	141.0 (120.0-167.0)	204.0 (167.0-241.0)	< 0.001
Cholesterol total (mg/dL)	163.0 (141.0-180.0)	171.0 (154.3-191.5)	0.06
Albumin (g/dL)	4.2 (4.0-4.4)	4.3 (4.2-4.6)	0.03

Data are shown as number of patients (percentage) or median value (25^{th}-75^{th} percentile), except for age (median and range). HCV: Hepatitis C virus; AST: Aspartate transaminase; ALT: Alanine transaminase.

Table 2 Association between angiopoietins and liver fibrosis in chronic hepatitis C patients

	n	Rho spearman coefficient	P value
Ang1	179	-0.297	5.25×10^{-5}
Ang2	179	0.402	2.37×10^{-9}
Ang2/Ang1	179	0.474	2.14×10^{-11}

Ang1: Angiopoietin 1; Ang2: Angiopoietin 2.

Diagnostic accuracy

Subsequently, receiver operating curve analyses were performed to demonstrate the diagnostic validity of each individual angiopoietin or combined as a ratio to classify liver fibrosis in CHC. As shown in Figure 2, both angiopoietins had a high power to differentiate patients with F > 1, F > 2 or cirrhosis. Indeed, the area under the curve (AUC) of Ang1 was 0.734, with a sensitivity 70.97% and a specificity 73.65% at its optimal cut-off for cirrhosis staging; likewise, Ang2 had an AUC of 0.761 for diagnosing CHC patients with cirrhosis, with a sensitivity 74.19% and a specificity 69.59% at the value corresponding to Youden index. Importantly, Ang2/Ang1 ratio displayed the highest precision in discriminating cirrhotic patients (Figure 2 and Table 3) with an AUC of 0.810, a sensitivity of 70.97% and a specificity of 84.46% at the optimal cut-off (10.33). Furthermore, the simultaneous analysis of both angiopoietins as a ratio greatly improves other clinically relevant parameters, such as positive likelihood ratio and accuracy (4.57 and 82.1, respectively). Although other cut-offs were inspected in order to improve sensitivity or specificity to 90% (data not shown), optimal criterion (Youden index) displayed better clinical results (Table 3).

Finally, the efficacy of Ang2/Ang1 for cirrhosis staging was compared with other previously described non-invasive serum markers [aspartate aminotransferase to alanine aminotransferase ratio (AAR), aspartate aminotransferase-to-platelet ratio index (APRI), fibrosis index based on the four factors (FIB4), fibrosis index (FI) and fibrosis-cirrhosis index (FCI)]. As Table 4 shows, angiopoietins ratio performs better than AAR (P = 0.01) and similar to the other indices (P > 0.05).

DISCUSSION

CHC is a major cause of progressive liver disease, which often leads to cirrhosis and HCC[37]. Monitoring of liver fibrosis is crucial for the clinical management of patients but its precise determination is only possible by histological examination of liver biopsies[28]. Since vascular remodelling has repeatedly been observed during the evolution of diverse CLD[13,34,35,38], the levels of main related factors, such as angiopoietins, might help to evaluate the progression of these diseases. Previous evidences suggested the possible pathogenic role of the Angiopoietins/Tie-2 system on cirrhosis development, thus highlighting its potential to detect the degree of liver injury[34,35,38]. In this regard, some reports described the significant elevation of Ang2 serum levels in patients with liver cirrhosis[39]. Pauta *et al*[40] also reported higher levels of Ang2 in the systemic and suprahepatic circulation of cirrhotic patients with alcoholic liver disease and established the inverse correlation of Ang1/Ang2 with prognostic models of the disease. Accordingly, our data indicate that circulating levels of angiopoietins in CHC patients are notably related to fibrosis. Moreover, a significant direct association between Ang2/Ang1 ratio and liver cirrhosis has also been observed. These findings

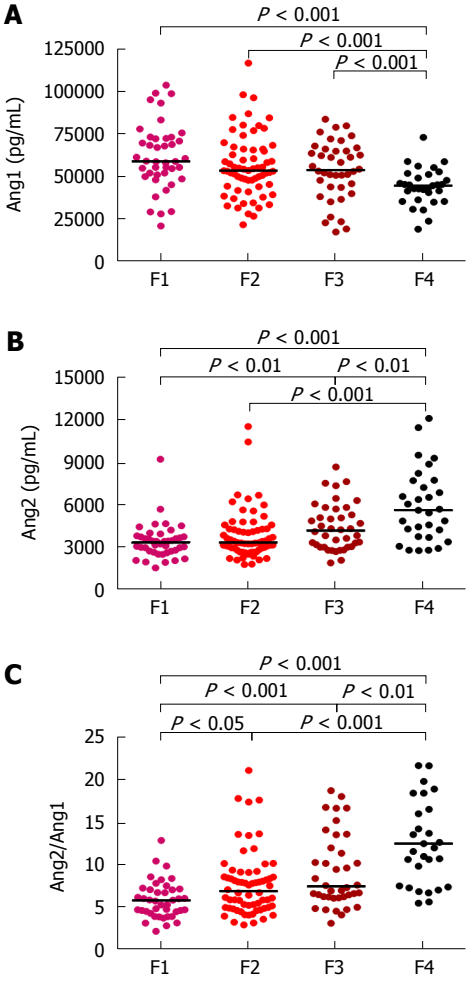

Figure 1 Distribution serum concentrations of Ang1 (A), Ang2 (B) and Ang2/Ang1 ratio values (C) in chronic hepatitis C patients. Medians are represented by horizontal lines. Statistical significance was tested using Mann-Whitney *U*-test. Ang1: Angiopoietin 1; Ang2: Angiopoietin 2.

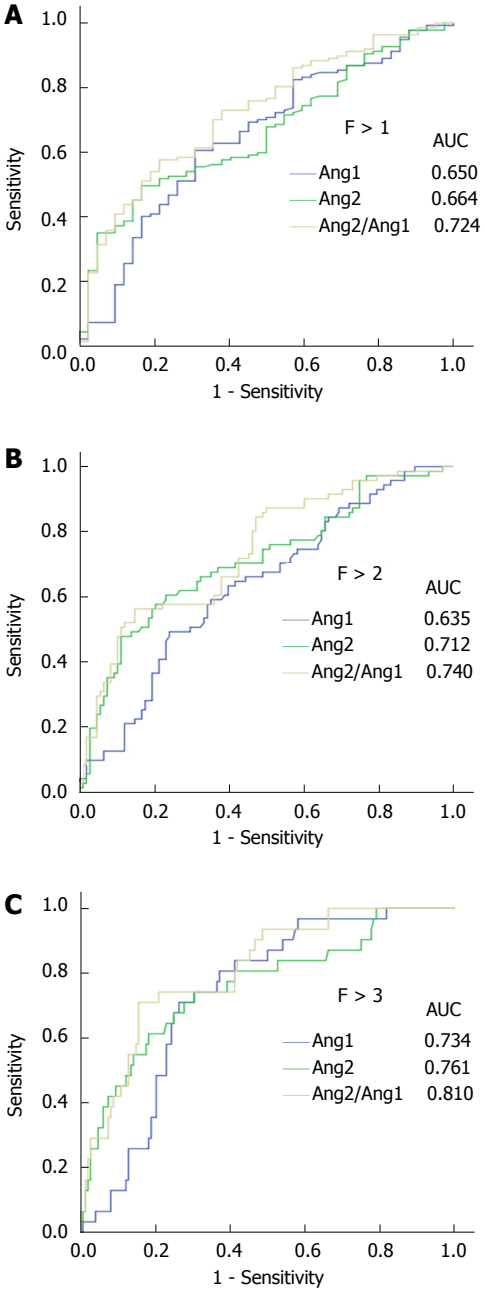

Figure 2 The receiver operating characteristic curve analysis on the abilities of Ang1, Ang2 and the Ang-2/Ang-1 ratio to diagnose. Significant fibrosis (F > 1) (A), advanced fibrosis (F > 2) (B) and cirrhosis (F > 3) (C) in patients with chronic hepatitis C. Ang1: Angiopoietin 1; Ang2: Angiopoietin 2.

concur with those of Vespasiani-Gentilucci *et al*[38] who reported a close relationship between fibrosis stage and peripheral levels of Ang1 and Ang2. Therefore, all these data highlight the useful role of these angiogenic factors as non-invasive markers of CHC progression. Furthermore, although ROC analysis revealed high accuracy of both, Ang1 and Ang2, (AUC-ROC > 0.7) to identify cirrhosis, Ang2/Ang1 ratio displayed the highest value of AUC-ROC (0.810) and showed valuable sensitivity and specificity for the diagnosis of cirrhosis.

In addition, it must be pointed out that Ang2/Ang1 ratio displays similar or superior precision than other proposed tests (AAR, APRI, FIB4, FI, and FCI). In spite the initial outstanding performance of recently defined index, enhanced liver fibrosis (ELF), which combines several variables (hyaluronic acid, tissue inhibitor of matrix metalloproteinases-1, and amino-terminal propeptide of type Ⅲ procollagen[41], the limited sample size of cirrhotic patients in that cohort (n = 29) could lead to overestimate its diagnostic potential[42]. Indeed, angiopoietins ratio displays better AUC-ROCs for cirrhosis identification when ELF is analyzed in a larger cohort of patients (0.81 *vs* 0.78). Finally, it must be noted that Ang2/Ang1 is also simplier and cheaper than other costly and undisclosed procedures such as FibroTest[43,44].

Taken together, these findings suggest that Ang2/Ang1 ratio might constitute a useful minimally invasive indicator of cirrhosis in CHC patients, which could notably help clinical decision-making during patient follow-up. However, the application of this novel biomarker in clinical practice might benefit from further validation.

Table 3 Accuracy of angiopoietins to discriminate significant fibrosis (F > 1), advanced fibrosis (F > 2) and cirrhosis (F > 3) in chronic hepatitis C patients

	AUC-ROC (95%CI)	Criterion	Se	95%CI	Sp	95%CI	+LR	95% CI	-LR	95%CI	+PV	95%CI	-PV	95%CI	Cost	ACC
F > 1																
Ang1	0.650 (0.575 -0.720)	≤ 54337.63	60.58%	51.9-68.8	69.05%	52.9-82.4	1.96	1.2-3.1	0.57	0.4-0.8	64.40	52.9-74.7	65.5	55.2-74.8	0.35	65.00%
Ang2	0.664 (0.589-0.732)	> 4041.67	49.64%	41.0-58.3	83.33%	68.6-93.0	2.98	1.5-6.0	0.60	0.5-0.7	73.30	60.1-84.1	64.2	55.0-72.7	0.33	67.20%
Ang2/ Ang1	0.724 (0.652- 0.788)	> 7.162	57.66%	48.9-66.1	78.57%	63.2-89.7	2.69	1.5-4.9	0.54	0.4-0.7	71.30	59.2-81.5	66.8	57.1-75.5	0.32	68.50%
F > 2																
Ang1	0.635 (0.560-0.705)	≤ 47573.25	49.3%	37.2-61.4	75.93%	66.7-83.6	2.05	1.4-3.1	0.67	0.5-0.9	41.80	28.7-55.9	81.0	73.0-87.5	0.31	69.00%
Ang2	0.712 (0.639-0.777)	> 4256	60.56%	48.3-72.0	76.85%	67.8-84.4	2.62	1.8-3.9	0.51	0.4-0.7	47.90	34.7-61.3	84.7	77.0-90.6	0.27	72.60%
Ang2/ Ang1	0.740 (0.669-0.802)	> 9.262	56.34%	44.0-68.1	85.19%	77.1-91.3	3.80	2.3-6.2	0.51	0.4-0.7	57.20	41.7-71.7	84.7	77.5-90.4	0.22	77.70%
F > 3																
Ang1	0.734 (0.663-0.797)	≤ 47573.25	70.97%	52.0-85.8	73.65%	65.8-80.5	2.69	1.9-3.8	0.39	0.2-0.7	37.90	25.9-51.2	91.8	85.3-96.1	0.27	73.20%
Ang2	0.761 (0.691-0.821)	> 4256	74.19%	55.4-88.1	69.59%	61.5-76.9	2.44	1.8-3.4	0.37	0.2-0.7	35.60	24.5-48.1	92.2	85.6-96.5	0.30	70.40%
Ang2/ Ang1	0.810 (0.744- 0.864)	> 10.33	70.97%	52.0-85.8	84.46%	77.6-89.9	4.57	2.9-7.1	0.34	0.2-0.6	50.90	35.8-65.9	92.8	87.0-96.5	0.18	82.00%

Youden index criterion. AUC-ROC: Area under the curve of receiver operator characteristics; Se: Sensitivity; Sp: Specificity; +LR: Positive Likelihood ratio; -LR: Negative Likelihood ratio; +PV: Positive predictive value; -PV: Negative predictive value; ACC: Accuracy, *n* = 179 chronic hepatitis C; Ang1: Angiopoietin 1; Ang2: Angiopoietin 2.

Table 4 Comparisons among area under the curve of receiver operator characteristics from angiopoietin-2/angiopoietin-1 ratio and other non-invasive cirrhosis indices

Indices	AUC-ROC (95%CI)	Standard error	*P* value
Cirrhosis			
Ang2/Ang1	0.810 (0.744-0.864)	0.040	-
AAR	0.643 (0.568-0.713)	0.050	0.010
APRI	0.887 (0.831-0.930)	0.025	0.106
FIB4	0.858 (0.798-0.906)	0.031	0.349
FI	0.805 (0.737-0.861)	0.047	0.995
FCI	0.750 (0.677-0.813)	0.053	0.372

Two-sided *P* values by De Long test. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AAR: AST to ALT ratio; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB4: Combination of biochemical values (platelets, ALT, AST) and age; FI: Fibrosis index; FCI: Fibrosis-cirrhosis index; AUC-ROC: Area under the curve of receiver operator characteristics; Ang1: Angiopoietin 1; Ang2: Angiopoietin 2.

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COMMENTS

Background

Hepatitis C virus (HCV) infection often progresses to chronic hepatitis C (CHC), one of the leading causes of cirrhosis and hepatocellular carcinoma (HCC). Angiogenesis is closely related to the pathogenesis of chronic liver disease and its progression to HCC.

Research frontiers

Several biomarkers have been investigated to predict liver cirrhosis in patients with CHC; however, few studies have evaluated the usefulness of angiogenic factors to identify cirrhosis in these patients despite angiogenesis often concurs with liver fibrosis.

Innovations and breakthroughs

This study shows that the peripheral value of angiopoietin-2 (Ang2)/angiopoitein-1 (Ang1) was significantly associated with liver fibrosis in patients with CHC, highlighting its potential as novel biomarker for the non-invasive diagnosis of liver fibrosis.

Applications

The laudable discriminatory accuracy displayed by Ang2/Ang1 for fibrosis staging might replace other complex and expensive test for monitoring CHC progression.

Terminology

The unbalance between Ang1 and Ang2 is present in many tumors such as HCC as well as in diverse chronic liver diseases underlining their potential pathogenic role and their impact as targets for therapeutic intervention.

Peer-review

This is a study regarding the role of Ang2/Ang1 ratio as a non-invasive biomarker of fibrosis in CHC. Overall, the manuscript was well-written and all tables and figures were appropriate. The main findings about Ang2/Ang1 ratio was quite novel. The idea of assessment of Ang2/Ang1 ratio as a noninvasive biomarker in cirrhosis in chronic hepatitis C is quite interesting.

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ORIGINAL ARTICLE

Basic Study

Transient receptor potential vanilloid 1-immunoreactive signals in murine enteric glial cells

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Author contributions: Yamamoto M conceived, designed and performed the *in vitro* cell culture experiments and immunocytochemistry, analyzed the data, and wrote the manuscript; Nishiyama M and Iizuka S bred the mice and prepared the specimens for further analyses; Suzuki S, Suzuki N and Aiso S contributed to experimental design and data analysis; Nakahara J conceived the study and designed and performed immunohistochemistry experiments; All authors read and approved the final version of the manuscript.

Institutional review board statement: This study was approved by the Institutional Review Boards of Keio University School of Medicine and Tsumura Research Laboratories.

Institutional animal care and use committee statement: All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Tsumura & Co. Ethical approval (Approval #14-088) for the experimental procedures used in this study was obtained from the Laboratory Animal Committee of Tsumura & Co. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: Technical appendix and dataset are available from the corresponding author at hirokoma@h.email.ne.jp.

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Abstract

AIM

To investigate the possible involvement of transient receptor potential vanilloid 1 (TRPV1) in maturation of enteric glial cells (EGCs).

METHODS

Immunohistochemical and immunocytochemical techniques were used to analyze EGC markers in myenteric plexus (MP) as well as cultured MP cells and EGCs using TRPV1 knockout (KO) mice.

RESULTS

We detected TRPV1-immunoreactive signals in EGC in the MP of wild-type (WT) but not KO mice. Expression of glial fibrillary acidic protein (GFAP) immunoreactive signals was lower at postnatal day (PD) 6 in KO mice, though the difference was not clear at PD 13 and PD 21. When MP cells were isolated and cultured from isolated longitudinal muscle-MP preparation from WT and KO mice, the yield of KO EGC was lower than that of WT EGC, while the yield of KO and WT smooth muscle cells showed no difference. Addition of BCTC, a TRPV1 antagonist, to enriched EGC culture resulted in a decrease in the protein ratio of GFAP to S100B, another EGC/astrocyte-specific marker.

CONCLUSION

These results address the possibility that TRPV1 may be involved in the maturation of EGC, though further studies are necessary to validate this possibility.

Key words: Enteric glial cells; Enteric nervous system; Glial fibrillary acidic protein; S100B; Smooth muscle cells

Core tip: We report that immunosignals of glial fibrillary acidic protein (GFAP) in myenteric ganglia in transient receptor potential vanilloid 1 (TRPV1) knockout (KO) mice are weaker than in wild-type mice in the early postnatal period, suggesting the possibility that the maturation of enteric glial cells (EGCs) might be retarded at least temporally in TRPV1 KO mice. Accordingly, in *in vitro* culture of isolated myenteric plexus cells/EGCs suggest that GFAP expression is affected by gene KO and an antagonist to TRPV1. The expression and function of TRPV1 in EGC merits further investigation.

Yamamoto M, Nishiyama M, Iizuka S, Suzuki S, Suzuki N, Aiso S, Nakahara J. Transient receptor potential vanilloid 1-immunoreactive signals in murine enteric glial cells. *World J Gastroenterol* 2016; 22(44): 9752-9764 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9752.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9752

INTRODUCTION

The enteric nervous system (ENS), an integrative neuronal network that resides within the gut wall, autonomously controls gastrointestinal (GI) motility, secretion and blood flow without major inputs from the brain[1,2]. The ENS is composed of two main cell types, neurons and enteric glial cells (EGC), the latter being several fold more abundant than neurons[3-5]. EGC share many phenotypical features with astrocytes, and were long believed to function mainly as support cells for neurons. However, emerging evidence has elucidated their regulatory role in a wide array of GI physiological aznd pathophysiological processes[6], including neurotransmission[7,8], motility[9-11], and inflammation[8], as well as in secretory/absorptive[12,13], barrier[8,14-16] and repair[17] functions of the intestinal epithelium and host defense against pathogens[18].

Transient receptor potential vanilloid receptor 1 (TRPV1) is a nonselective cation channel activated by exogenous plant-derived vanilloid compounds such as capsaicin and resiniferatoxin, as well as by endogenous membrane-derived lipid endocannabinoids such as anandamine, 2-arachidonoyl-glycerol and N-arachidonoyl-dopamine[19]. Moreover, TRPV1 is known to be a transducer channel activated by high temperature, low pH and mechanical/osmotic stimuli. Although attention has been directed mainly to sensory neurons as the site of TRPV1 localization, TRPV1 expression has been detected in non-neuronal tissues/cells, including keratinocytes of the epidermis, bladder urothelium, smooth muscles, liver, polymorphonuclear granulocytes, mast cells and macrophages[19].

TRPV1 has been reported to be present in astrocytes in brain[20], spinal cord[21] and retina[22], and possibly to be involved in glial activation[23], cell migration[24], amyloid-β-induced inflammation[25] and traumatic brain injury[26]. However, it is unknown whether TRPV1 is present and functional in enteric glia. In the present study, using TRPV1-deficient [knockout (KO)] mice and an acid-ethanol fixation protocol, specific TRPV1-immunoreactive (TRPV1-IR) signal was detected in wild-type (WT) EGC. In addition, the possible involvement of TRPV1 in the differentiation of EGC was investigated.

MATERIALS AND METHODS

Antibodies

Details of the primary antibodies used in the present study are shown in Table 1. The specificity of anti-TRPV1 antibodies is presented in Supplementary Figures S1 and S2. The secondary antibodies used were FITC-labeled donkey anti-mouse IgG antibody and Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, United States) for intestinal tissues and Alexa488-conjugated goat anti-mouse antibody and Alexa568-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, United States) for isolated longitudinal muscle layer-myenteric plexus (LM-MP) and cultured cells.

Animals

C57Bl/6 mice were from Charles River Laboratories Japan Inc. (Kanagawa, Japan). TRPV1-deficient (B6.129X1-Trpv1<tm1Jul>/J) mice originally obtained from Jackson Laboratories (Bar Harbor, ME, United States) were maintained at Charles River Laboratories Japan Inc. and transported to the animal facilities of Tsumura Laboratories on gestational day 14 (dams, 1

Table 1 Summary of the primary antibodies used in this study

Antibody	Provider	Source and type	Product/clone number	Immunogen	Usage
TRPV1	Bios	Rabbit polyclonal	bs-1931R	a.a.825-835 of human TRPV1 (EDAEVFKDSMAPGEK)	Figures 1-3, Suppl. Figure 1
	LifeSpan	Mouse monoclonal	LS-C122800	a.a. 819-835 of rat TRPV1 (CGSLKPEDAEVFKDSMVPGEK)	Figure 6, Figure 7, Suppl. Figure 1
GFAP	BD	Mouse monoclonal	cocktail of 4A11, 1B4 and 2E1	cow spinal cord homogenate (4A11, 1B4) or human /bovine GFAP (2E1)	Figures 1-3, Suppl. Figure 1
	DAKO	Rabbit polyclonal	Z0344	isolated GFAP from cow spinal cord	Figure 6, Figure 7
	CST	Mouse monoclonal	GA5	isolated GFAP from pig spinal cord	Suppl. Figure 2
PGP9.4	Abcam	Guinea pig serum	ab10410	a.a. 175-191 of human PGP9.5 (GASSEDTLLKDAAKVCL)	Figure 6, Figure 7
S100β	Proteintech	Rabbit polyclonal	15146-1-AP	recombinant human S100β	Figure 7, Figure 8, Suppl. Figure 3
αSMA	Novus	Rabbit monoclonal	E184	synthetic peptides corresponding to N-terminus (human)	Suppl. Figure 2

The negative controls of immunostaining performed in this study are shown in Supplementary Figure S6. Corresponding sequence of mouse TRPV1: TGSLKPEDAEVFKDSMAPGEK. The amino acid underlined is different from rat and the amino acid double-underlined is different from human. TRPV1 KO mice used in the present study preserved this sequence but the signal of immunohistochemistry in KO mice is faint, even in case detected, compared to that in WT mice, in the same staining and signal development protocols on the same slide on which the specimens from KO and WT mice were mounted together. Similar observations have been reported by Yamada *et al*, J Histochem Cytochem 2009; 57: 277-287.

dam per cage) or at the age of 7 wk (adult males, 4 mice per cage). The animals were allowed free access to water and standard laboratory food, and were housed at a temperature of 23 ± 2 ℃ with relative humidity of 55% ± 10%, and a 12:12-h light/dark cycle (with lights on from 07: 00 to 19: 00 daily).

All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Tsumura & Co. Ethical approval for the experimental procedures used in this study was obtained from the Laboratory Animal Committee of Tsumura & Co. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Sample collection

For the preliminary screening, 2 TRPV1-deficient (KO) and 2 C57/Bl6 WT dams were used. A total of 2 pups (each pup isolated from different dams) were randomly selected and sacrificed at each time point for both the KO and WT groups. Samples were collected on the day of birth (PD 0) and at around postnatal day 10 (PD 10-12), 20 (PD 20-21), 30 (PD 30-33), 60 (PD 61-62) and 90 (PD 91-92). After anesthesia with isoflurane, animals were transcardially perfused with ice-cold normal saline followed by ice-cold acid-ethanol solution (a mixed solution of ethanol and acetic acid at a ratio of 20:1 v/v) to fix the tissues. Large and small intestines (LI and SI, respectively) were dissected and further fixed in acid-ethanol solution overnight at 4 ℃, then cryoprotected and embedded in O.C.T. Compound (Sakura Finetech, Tokyo, Japan) for frozen sectioning according to standard procedures. For the confirmation analysis, 6 KO and 6 WT dams were used. A total of 6 pups (each pup isolated from different dams) were sacrificed at PD 6, PD 13 and PD 21. Intestinal tissue samples were collected as described above.

Immunohistochemistry of intestinal tissue sections

Ten μm-thick frozen sections were incubated in phosphate-buffered saline (PBS) for 10 min at room temperature, and then incubated with a mixture of primary antibodies overnight at 4 ℃. After a thorough wash with PBS, sections were incubated with a mixture of secondary antibodies with nuclear counterstain (TO-PRO3; Molecular Probes) for 1 h at room temperature, washed and finally mounted with Vectashield (Vector, Burlingame, CA, United States). Sections were observed and digital images were recorded with a confocal laser scanning microscope (C-1; Nikon, Tokyo, Japan).

IHC of whole-mount preparation of LM-MP

LI segments were isolated from 5-wk-old mice and the LM-MP was peeled off. The peeled LM-MPs were stretched taut, pinned flat to a silicone ring and fixed with ice-cold acetone for 30 min. After fixation, each preparation was washed 3 times for 10 min each in PBS. The preparations were placed in Superblock (Thermo Fischer Scientific, Rockford, IL, United States) containing 0.3% Triton X-100 overnight at 4 ℃. The preparations were then placed in a mixture of primary antibodies diluted in antibody diluent (DAKO Japan, Tokyo, Japan) overnight at 4 ℃. After removal from the primary antibody, the tissues were rinsed 3 times for 10 min per rinse with PBS and then incubated with a mixture of the relevant secondary antibodies overnight at 4 ℃. After a final set of rinses, the preparations were mounted on microslides and coverslipped with Prolong Gold antifade reagent (Molecular Probes). The slides were observed using confocal laser microscopy FV-100D (Olympus, Tokyo, Japan).

Co-culture of myenteric plexus cells and smooth muscle cells

We prepared MPC/SMC mixture from SI, because a far smaller number of MPC/SMC were obtained from LM-MP of LI, presumably due to the short length of the LI tract and inefficient cellular liberation from the tissue. SI segments were isolated from 5-wk-old WT and KO mice, and the LM-MP was peeled off. LM-MP was digested in digestion buffer containing 0.1% type II collagenase and 0.1% soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, United States) at 31 ℃ for 30 min. The buffer was then replaced with new digestion buffer and the mixture incubated at 31 ℃ for an additional 30 min. The remaining tissue pieces were dissociated by mechanical shearing through micropipette tips. The cells were spun down at 200 g for 5 min and suspended in HuMedia-SG2 (Kurabo, Osaka, Japan). The cells were plated in type IV-collagen coated plates (BD Biosciences, San Jose, CA, United States). The medium was replaced on the next day, which led to discarding of almost all of the cells. The remaining attached cells, which were round and small, began to proliferate discernibly by around day 3. The cells were cultured for an additional 8 d. A representative image of the cells is shown in Supplementary Figure S3. Analysis by phase-contrast microscopy showed no apparent difference in the number and appearance of the cells between cultures derived from WT and KO mice.

Enriched EGC culture

Co-culture of myenteric plexus cells and smooth muscle cells (SMCs) was initiated as described in the previous section. On day 5 of the co-culture, the cells were trypsinized, washed and labeled with anti-NGF receptor p75 rabbit polyclonal antibody[27] for 5 min on ice. The cells were then washed and incubated with biotinylated anti-rabbit IgG antibody (BD Biosciences) for 5 min on ice. The cells were subsequently washed and mixed with magnetic beads conjugated with streptavidin. MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used to enrich NGF-receptor p75-positive cells, which are derived from the neural crest. The cells were plated on poly-D-lysine-coated plate and cultured for an additional 6 d. Enriched EGC culture was performed using WT mice only, because the culture protocol has been found to be inapplicable to KO mice (the resultant culture contained only a trace number of surviving cells).

Immunocytochemistry (IHC) for enriched EGC cultures

The cells were fixed with 4% phosphate-buffered paraformaldehyde for 15 min. The cells were then washed twice with PBS and permeabilized in 0.3% Triton-100 in PBS for 15 min. After being rinsed with PBS, sections were incubated overnight in a mixture of the primary antibodies, after which the cells were incubated in a mixture of the relevant secondary antibodies (1:1000; Molecular Probes) for 60 min at room temperature. For TRPV1-staining, biotin-labeled anti-mouse IgG (BD Biosciences) and streptavidin-conjugated Alexa Fluor 647 (Molecular Probes) were used to amplify the signal intensity. Nuclei were stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI; Life Technologies, Carlsbad, CA, United States). The cells were visualized, photographed and analyzed using Celaview RS100 (Olympus) or Image Xpress (Molecular Probes) cell imaging systems.

Imaging analysis for IHC

In IHC from intestinal sections, the green fluorescence of glial fibrillary acidic protein (GFAP) in MP was detected and quantitated using Image J image analysis software (version 1.40g; National Institute of Health, Bethesda, MD, United States, http://rsbweb.nih.gov/ij/)[28]. The fluorescence intensity was normalized to the circumferential length of the intestinal tract.

Imaging analysis for MPC and SMC co-culture system

The MPC and SMC co-culture system contained a large number of α smooth muscle actin (αSMA)-positive cells (*i.e.,* SMC) and a small population of $GFAP^+$ cells and $GFAP^-\alpha SMA^-$ cells. Virtually no $PGP9.5^+$ cells were detected. After eliminating $GFAP^-\alpha SMA^-$ cells by imaging analysis, αSMA^+ SMC and $GFAP^+$ cells were easily distinguished by DAPI fluorescence; SMC have large nuclei with weak DAPI staining and EGC have small nuclei with bright DAPI staining (Supplementary Figures S3 and S4). We counted the number of small bright nuclei and large dim nuclei separately by setting the gate for size and brightness of the nucleus image (Supplementary Figure S4). The number of eliminated cells was then counted. The percentage of $GFAP^+$ cells and SMC was defined as the ratio of the number of the cells in the respective populations to the total number of total MPC (DAPI-stained cells).

Imaging analysis for enriched EGC culture

In enriched EGC culture, the majority of SMCs was eliminated and most cells (> 90%) were stained with anti-GFAP, anti-S100B, or both antibodies. GFAP was stained with mouse monoclonal antibody and visualized with Alexa488-conjugated anti-mouse IgG. S100B was stained with rabbit polyclonal antibody and visualized with Alexa568-conjugated anti-rabbit IgG using Celaview (Supplementary Figure S5). For quantitation of fluorescence, we used a different laser source with a different wavelength (excitation wavelengths of 488 and 568 nm, respectively) through different band path filters under different exposure settings optimal for each fluorescence. Therefore, it was not possible to directly compare the fluorescence intensity values that had been obtained using different methods and measuring rules. As a result, in this analysis, we measured the fluorescence of GFAP and

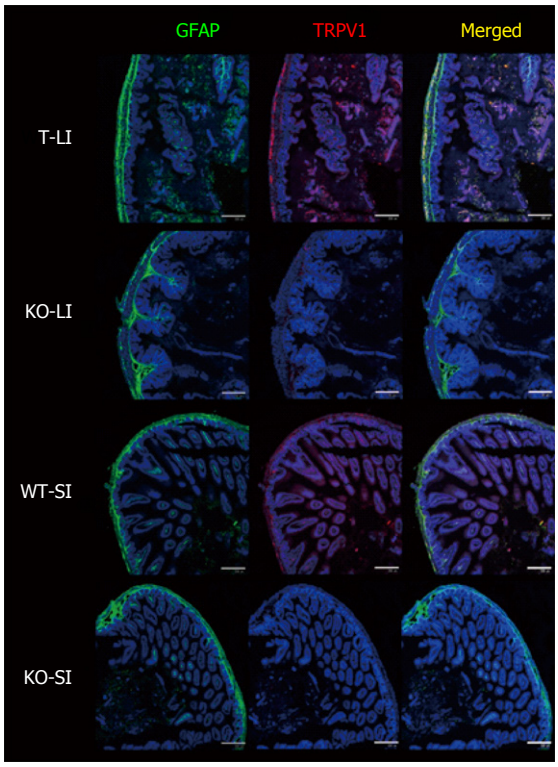

Figure 1 Double-immunostaining with GFAP and TRPV1 of small and large intestines of wild-type and transient receptor potential vanilloid 1-deficient mice. The degree of GFAP-IR signal observed in the enteric nervous system was similar in WT and KO mice. TRPV1-IR signal was observed only in WT mice and was located in a confined area of the smooth muscle layer. Nuclei were stained with TO-PRO3. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 200 μm. TRPV1: Transient receptor potential vanilloid 1; SI: Small intestine; LI: Large intestine; WT: Wild-type; KO: Knockout; GFAP: Glial fibrillary acidic protein.

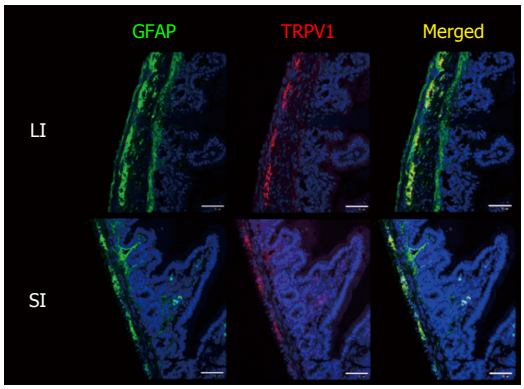

Figure 2 Enlarged view of GFAP/TRPV1-stained small intestine and large intestine of wild-type mice. Certain parts of Figure 1 were magnified. GFAP is located in the myenteric plexus, submucosal plexus and fibrous structures penetrating into the circular muscle layer and mucosal layer (in WT LI, the serosal membrane was also stained with GFAP; however, serosal membrane is known to show false-positive immunosignals with various antibodies). The location of the TRPV1-IR signal coincided with some portions of the GFAP-IR signal in the myenteric plexus. Nuclei were stained with TO-PRO3. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 50 μm. GFAP: Glial fibrillary acidic protein; TRPV1: Transient receptor potential vanilloid 1; SI: Small intestine; LI: Large intestine; WT: Wild-type.

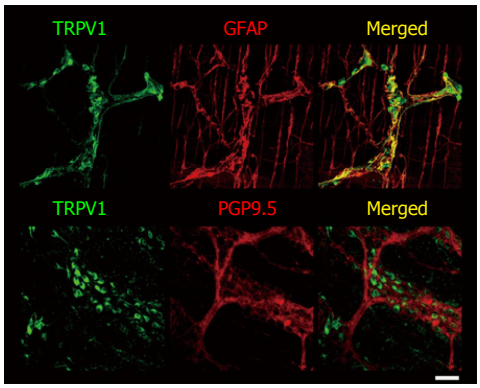

Figure 3 Double-immunostaining with TRPV1 and GFAP or PGP9.5 of large intestine longitudinal muscle layer-myenteric plexus of wild-type mice. LM-MP was isolated from 5-wk-old mice and whole-mount immunohistochemistry was performed as described in Materials and Methods. TRPV1 stained both cell bodies and fibers, while GFAP stained mainly fibers and some portions of their IR signals were co-localized. Co-localization of PGP9.5 and TRPV1-IR signals was not observed. Nuclei were stained with DAPI. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 50 μm. TRPV1: Transient receptor potential vanilloid 1; GFAP: Glial fibrillary acidic protein; LM-MP: Longitudinal muscle layer-myenteric plexus; WT: Wild-type.

S100B separately comparing the BCTC-treated wells and control wells. These cells had been isolated from a single preparation and dispensed into the wells at the same density.

Statistical analysis

Data are expressed as mean ± SEM. Unpaired Student's *t*-test was used to analyze differences between 2 groups. For comparison among 3 groups, the Dunnett test was performed. $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Expression of TRPV1 and GFAP was analyzed in LI and SI, of WT and KO young adult mice, by IHC (Figure 1). While a similar level of GFAP-IR signals was detected in both WT and KO mice, TRPV1-IR signal was abolished in KO mice. Several antibodies against TRPV1, including both monoclonal and polyclonal antibodies, gave essentially the same result (2 examples of which are shown in Supplementary Figures S1 and S2). In magnified view, TRPV1-IR signals were detected in a population of $GFAP^+$ cells (*i.e.,* EGC) mainly within the MP in both LI and SI (Figure 2). These data suggest that, at least some fractions of type Ⅰ (intraganglionic) and/or type Ⅱ (interganglionic) EGC[5] in the MP express TRPV1. The observation was further supported by high magnification IHC using isolated LM-MP. As shown in Figure 3, TRPV1-IR signal co-existed with GFAP-IR signals but not with the enteric neuron marker PGP9.5-IR.

To investigate the functional role of TRPV1 in the

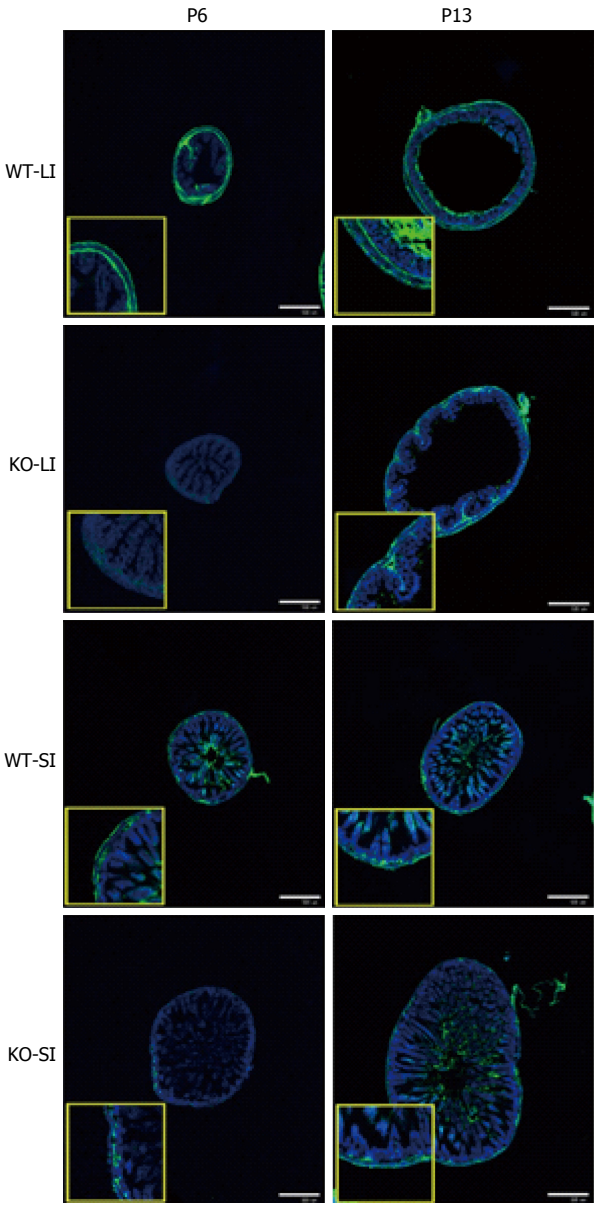

Figure 4 Difference of glial fibrillary acidic protein immunosignals between wild-type and knockout mice (image). Large intestine (LI) and small intestine (SI) samples were isolated from WT and KO mice on postnatal day (PD) 6 and PD 13. While the GFAP-IR signal was similar in WT and KO mice on PD 13, the signal on PD 6 was weaker in KO mice than in WT mice, both in SI and LI. Nuclei were stained with TO-PRO3. Representative data of an experiment using 6 mice per each time point/group. Scale bar represents 500 μm. GFAP: Glial fibrillary acidic protein; WT: wild-type; KO: Knockout.

postnatal development of EGC, we screened a series of LI and SI samples both from WT and KO mice at various postnatal days ranging from PD 0 to PD 92 by visual inspection. Only the samples obtained at PD 5 showed an apparent difference in GFAP-IR signals between WT and KO mice. Accordingly, image analysis was used to quantitate GFAP-IR signals at PD 6, PD 13 and PD 21 in WT and KO mice (n = 6 per time point). The results revealed that GFAP-IR signals at PD 6 were significantly weaker in KO mice than in WT, both in LI and SI; however, this difference was not observed at PD 13 nor PD 21 (Figures 4 and 5).

To obtain information about the possible influence of TRPV1 on EGC, we subsequently performed experiments using a co-culture system with MPC and SMC. Expression of GFAP and αSMA protein started at around culture day 5. At the end of the culture period, as described in Materials and Methods, most of the cells were αSMA^+ SMC and the remainder was $GFAP^+$ cells and αSMA^- $GFAP^-$ cells. The results are in good accordance with those of previous studies[29-32].

Using the same digestion solution and preparation procedure as described above, cells were prepared from LM-MP of WT and KO mice simultaneously. The cells were cultured for 11 d and the number of αSMA^+SMC, $GFAP^+$ cells and αSMA^-GFAP^- cells was counted and represented as the ratio of the number of each cell population to the total cell number. The experiment was repeated 4 times; the results are summarized in Figure 6. The yield of $GFAP^+$ cells was significantly lower in KO than WT mice, while the yield of SMC was not.

Enriched EGC were prepared from the 5-d co-cultures as described in the Materials and Methods, and the resultant culture contained EGC as the major cell population and a smaller population of SMC. The IHC results for the enriched EGC culture are shown in Figure 7. TRPV1-IR signal was present in $GFAP^+$ and/or $S100B^+$ cells but not in αSMA^+ cells.

Finally, we examined the effect of the TRPV1 agonist capsaicin and the TRPV1 antagonist BCTC on the expression of glial markers in enriched EGC cultures (Figure 8). GFAP-IR signals, but not S100B-IR signals, were significantly decreased by BCTC at 3 μmol/L. Capsaicin at concentrations of 33, 100, 3000 and 10000 nmol/L had no effect (data not shown).

DISCUSSION

Many researchers have reported the presence of functional TRPV1 in the sensory nerves of the GI tract[33-36] and some have also reported its presence in intrinsic enteric nerves of the MP[37-39]. It is controversial as to whether TRPV1 exists in GI structures other than extrinsic nerves, apart from in infiltrating inflammatory cells[40,41]. The immunostaining pattern shown in the present study resembled that of previous studies[37-39], demonstrating TRPV1-IR signals in intrinsic enteric nerves of the MP of guinea pig SI and LI. Thus, to our knowledge, our study is the first and only report addressing the possible presence of TRPV1 in EGC. The specificity of the antibodies used in this study was validated using TRPV1 KO mice (Figures 1 and 2) and recombinant TRPV1-expressing cells (Supplementary Figure S2). There are several possible reasons for the apparent discrepancies between our results and those of previous studies.

Firstly, the different antibodies may specifically detect different forms of TRPV1 protein. Buckinx *et al*[39]

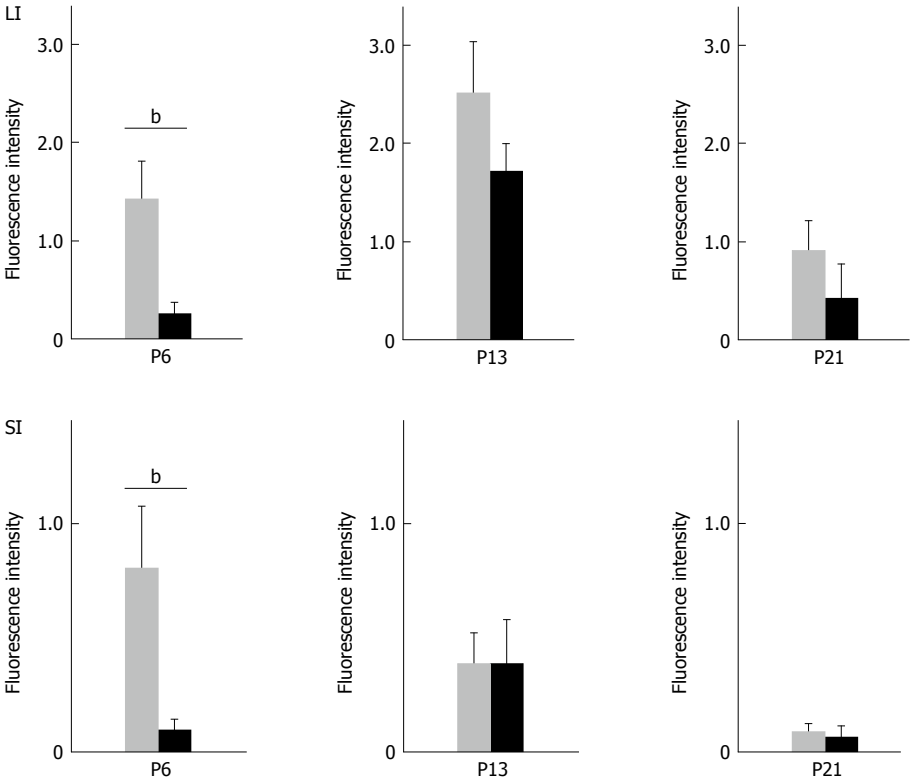

Figure 5 Difference in glial fibrillary acidic protein-IR signals between wild-type and knockout mice. The intensity of GFAP-IR signal for WT and KO mice on postnatal day (PD) 6, PD 13 and PD 21 was quantitated by imaging analysis as described in Materials and Methods. The amount of GFAP-IR fluorescence was normalized to the circumferential length of the intestinal tract. Data represent mean ± SEM, n = 5-6. $^{b}P < 0.01$. GFAP-IR: Glial fibrillary acidic protein; WT: Wild-type; KO: Knockout.

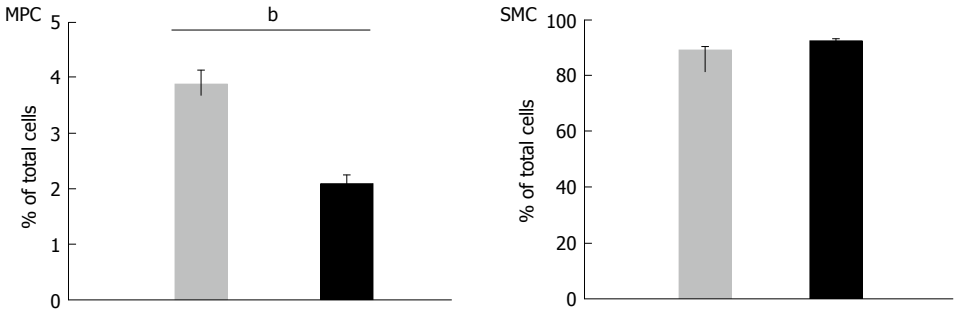

Figure 6 Specific decrease in the number of myenteric plexus cells in knockout mice. The cells were prepared from longitudinal muscle layer-myenteric plexus (LM-MP) of wild-type (WT) and KO mice simultaneously, using the same digestion solution in a single preparation. After 11-d culture, the numbers of α smooth muscle actin (αSMA)+ smooth muscle cells (SMCs), glial fibrillary acidic protein (GFAP)$^{+}$ cells and αSMA^{-}GFAP^{-} cells were determined as described in Materials and Methods. Nuclei were stained with DAPI. The percentage of GFAP^{+} cells and SMCs was calculated as the ratio of the number of each cell population to the number of total DAPI^{+} cells. The yield of enteric glial cells (EGCs, GFAP+ cells) in KO mice was significantly lower than that in WT mice, while the number of SMCs obtained was similar for both types of mice. Data represent mean ± SEM, n = 4. $^{b}P < 0.01$. KO: Knockout.

have reported that a guinea pig and a rabbit antibody raised against slightly different regions of the C-terminus of mouse TRPV1 yielded different staining patterns; *i.e.,* the former stained cytosolic IR signals and the latter stained fibrous IR signals. The antibodies used in the present study appeared to stain both cell bodies and fibers. TRPV1 protein is suggested to be present and functional in the cell membranes as well as intracellular organelles such as the endoplasmic reticulum, Golgi bodies and mitochondria[42-48]. These different staining patterns might therefore result from conformational differences between intracellular and plasmalemmal TRPV1. Differences in interacting molecules also influence epitope recognition by antibodies. It should be noted the C-terminal region of TRPV1 contains several modulatory regions, such as phosphorylation sites and binding sites for calmodulin and phosphatidylinositol 4,5-bisphosphate[49,50].

Secondly, we used acid ethanol fixation while the above-mentioned studies used methanol, Zamboni's

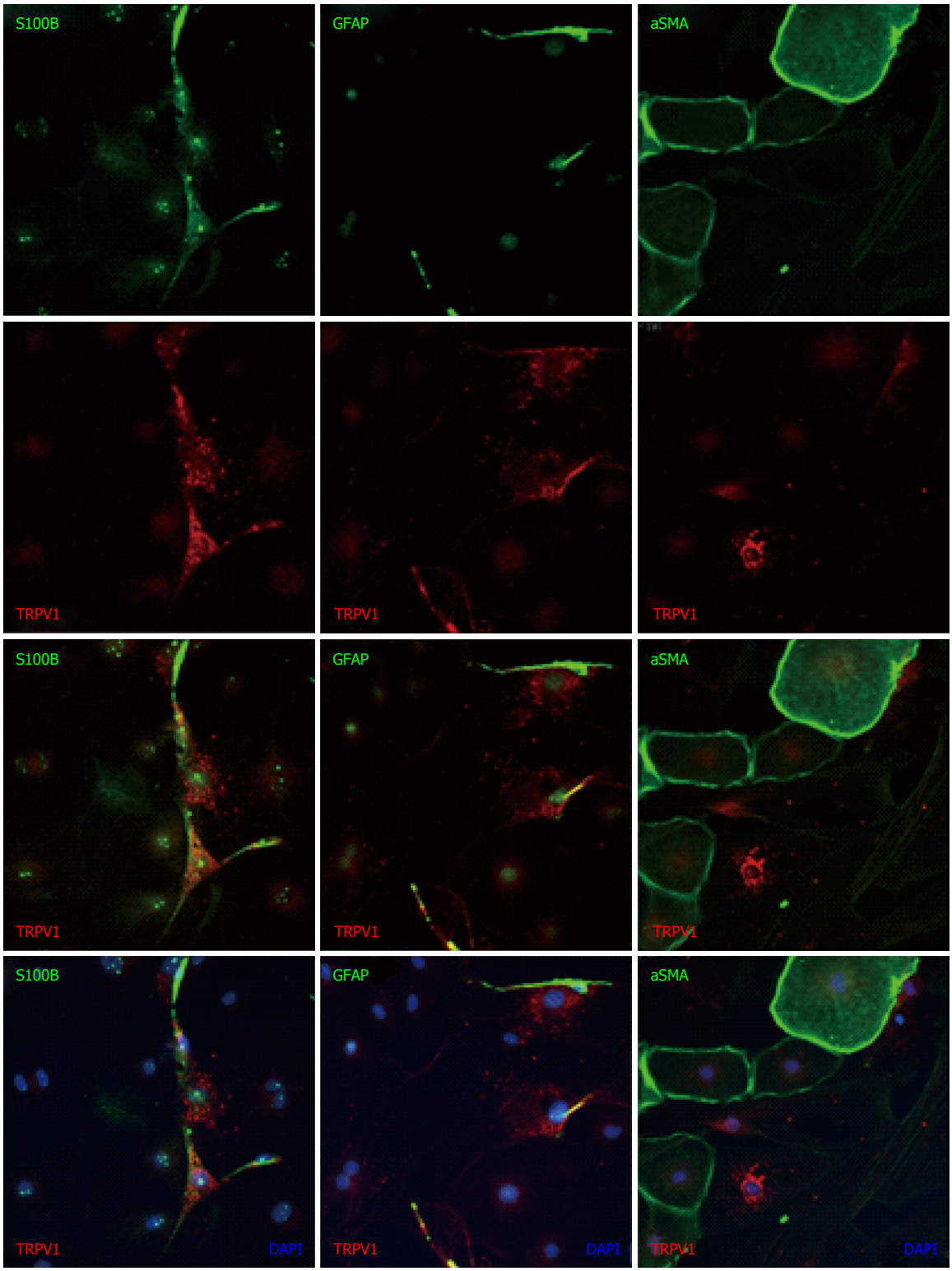

Figure 7 Immunostaining of transient receptor potential vanilloid 1 in enriched enteric glial cell culture. EGCs were isolated and cultured as described in Materials and Methods. The cells were labeled with antibodies to glial fibrillary acidic protein (GFAP), S100B or smooth muscle cell (SMC). The cultures contained many EGCs and a small percentage of SMCs. The location of TRPV1-IR signal coincided with that of the GFAP-IR signal and the S100B-IR signal, but not with that of the αSMA-IR signal. Nuclei were stained with DAPI. Scale bar represents 20 μm. TRPV1: Transient receptor potential vanilloid 1; EGC: Enriched enteric glial cell.

and paraformaldehyde fixation. IHC of certain glial proteins has been known to provide different results depending on the fixation procedure. For example, certain anti-GFAP antibodies were reported to detect mainly fibrous astrocytes in brain white matter after acid-alcohol fixation, while protoplastic astrocytes are detected in brain grey matter after aldehyde-based fixation[51]. Comparison of the intensity of IHC with

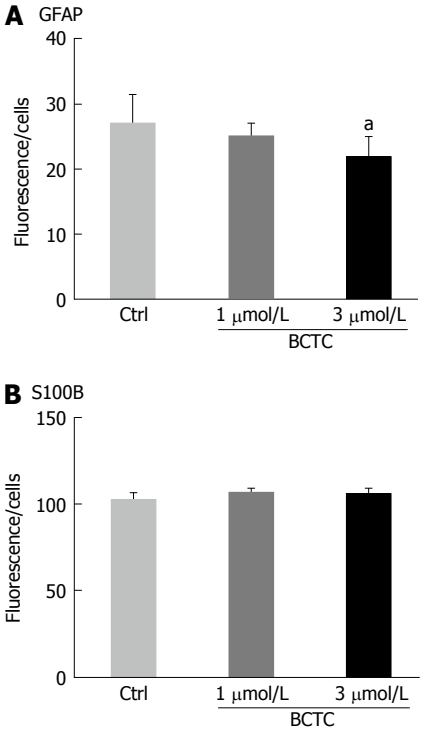

Figure 8 Effect of transient receptor potential vanilloid 1 agonist/antagonist on the signal ratio of glial fibrillary acidic protein-IR to S100B-IR. Enriched enteric glial cells [EGCs; prepared from day 5 myenteric plexus cells (MPCs) and smooth muscle cells (SMCs) co-culture] were plated and cultured for an additional 5 d. The TRPV1 antagonist BCTC was added and after 24 h the cells were fixed. The cells were stained with antibodies and imaging analysis was performed using a Celaview system as described in Materials and Methods. BCTC (3 μmol/L) decreased the GFAP-IR signal but the S-100B-IR signal was unchanged. Data represent mean ± SEM, n = 5-8. $^{a}P < 0.05$. TRPV1: Transient receptor potential vanilloid 1; GFAP: Glial fibrillary acidic protein.

the amount of GFAP protein estimated by enzyme-linked immunosorbent assay has suggested that ethanol-based fixation provides better results than aldehyde-based fixation[52]. Vimentin in astrocytes and Bergmann glial fibers in cattle, rabbit and rat brain[53], and P-glycoprotein in rat brain astrocytes[54] were also reported to be detected only after ethanol-acid fixation. Along these lines, in the course of our research on EGC ontogeny, we have found that acid-ethanol fixation provides stronger and more specific IHC for GFAP in EGC of the mucosal and smooth muscle layers of SI and LI.

Thirdly, it should be noted that the present data does not exclude the possibility of the presence of TRPV1 in enteric nerves. We did not detect the co-existence of TRPV1-IR signals with PGP9.5-IR signals, but this might have been due to the relatively poor sensitivity and resolution of the immunostaining patterns of PGP9.5 in the present study. Furthermore, previous studies demonstrating the existence of TRPV1-IR signals in intrinsic myenteric neurons used antibodies to choline acetyltransferase, calbindin and calretinin[37,38]. Matsumoto *et al*[40] also reported that TRPV1-IR did not co-localize with PGP9.5 and NeuN in cell bodies of the MP. We found immunohistochemically-stained $TRPV1^{+}GFAP^{-}$ areas in the LM-MP. Because the GFAP antibody appeared to mainly stain fibrous structures inside the cells, the $TRPV1^{+}GFAP^{-}$ area could represent EGCs. However, it is possible that these areas are contained in structures other than EGCs. Extensive research, including studies on cellular and/or intracellular TRPV1-mediated calcium mobilization in these cell types, will be needed to clarify these issues.

TRPV1 has been reported to be expressed by astrocytes in mouse spinal cord[21], in mouse, rat and human brain[20,23,55], in rat retina[22], and in *in vitro* cultured rat astrocytes[56]. Treatment with the TRPV1 agonist resiniferatoxin was reported to increase Fos expression by astrocytes in mouse brain. Furthermore, injection of capsaicin, another TRPV1 agonist, led to an increase in markers for microglia (ionized calcium-binding adapter molecule 1, Iba1) as well as astrocytes (GFAP) in the dorsal horn of the spinal cord after adjuvant-induced arthritis or partial sciatic nerve ligation[23], and in the trigeminal nucleus caudalis[57]. Treatment with TRPV1 antagonist decreased the migration of reactive astrocytes isolated from the wounded retina[24]. These data suggest that TRPV1 stimulation resulted in the activation of astrocytes and, presumably, microglia. TRPV1 in astrocytes has also been suggested to be involved in the pathogenesis and epileptogenesis of human mesial temporal lobe epilepsy[55]. These reports suggest that TRPV1 in astrocytes is functional and plays certain roles in astroglial biology.

In the present study, firstly, TRPV1 KO mice showed weaker GFAP-IR signals only at PD 6, but not at PD 13 nor PD 21. Secondly, the number of GFAP-expressing cells developed from the isolated MPC was significantly lower in TRPV1 KO mice than in WT mice. Thirdly, treatment of isolated WT MPC with the TRPV1 antagonist BCTC resulted in a decrease in the expression ratio of GFAP to S100B; the latter is another frequently-used EGC/astrocyte-specific marker[58]. S100B is a diffusible Ca^{2+}/Zn^{2+}-binding protein that is considered to be a "janus face" neurotrophin for neuron and astrocytes[59] and to act as a proinflammatory cytokine involved in gut inflammation with specific relevance to nitric oxide production[60]. It has been speculated that the intensity and differential intracellular localization of GFAP-IR and S100B-IR signals is related to the degree of differentiation and/or functional diversity of astrocytes[39,61-64]. These data suggest that TRPV1 signaling may interfere with GFAP expression in EGC, at least during a certain period of EGC maturation.

Because GFAP is widely recognized as an astrocyte differentiation marker, constituting the major intermediate filament protein of mature astrocytes[65-67], the present finding suggests that TRPV1 might be involved in the differentiation/maturation of EGC. The ENS

originates in the neural crest, which invades, proliferates and migrates within the intestinal wall until the entire bowel is colonized with enteric neural crest-derived cells (ENCDCs)[68]. After initial migration, ENCDCs differentiate into glia and neuronal subtypes and form a critical constituent for nervous system function. Although little is known about mechanisms controlling the development and differentiation of EGC, it has been suggested that Sox-10, Lgl4, ADAM22 and bone morphogenetic proteins are involved[4]. GFAP appears later at the end of the mouse embryonic stage. Cells with functions similar to those of ENCDCs exist in the bowel of adult and newborn humans and rodents[69,70]. The present data indicate that TRPV1 might be involved in the regulation of GFAP expression in *in vitro* cultured MPC prepared from young adult animals as well as *in vivo* in the early postnatal period (around PD 6). Although the present study did not examine ENCDC markers, methods similar to ours have been used to obtain enteric neural stem cells for enteric neural stem cell therapy[71,72].

The following three points, however, should be noted. Firstly, we did not examine the functions of EGC. As reported for central nervous system astrocytes, deletion or alteration of TRPV1 signaling might influence EGC function. Further intensive investigation will be needed to clarify this point. Secondly, although a normal microenvironment around EGC can compensate for the effect of TRPV1 deletion, at least until PD 10 in the case of diseased intestines (*e.g.,* due to damage of neurons, SMCs and mucosa, the effect of TRPV1 deficiency might be more severe because of a lack of compensatory signals). Along these lines, we could not obtain enriched EGC from TRPV1 KO mice as described in the Methods section. Because enriched EGC cultures contain far smaller numbers of SMC compared to unenriched MPC culture, the signals from SMC may be important for compensation. Additional studies are needed to determine the possible effects of TRPV1 deletion on diseased intestine, such as intestinal inflammation, hyperalgesia after psychological or surgical stress, and functional dysregulation (*e.g.,* dismotility, nutrient malabsorption and diarrhea/constipation). It is possible that TRPV1 plays an important role in the repair/recovery/restore stage of GI tissues. Finally, the present study suggested the possible existence of TRPV1 in EGC mainly by immunohistochemical/immunocytochemical techniques. Biochemical isolation and identification of TRPV1 protein and functional validation of TRPV1, such as through agonist-specific Ca^{2+} influx and Na^{+} current, are necessary to establish the existence and function of TRPV1 in EGC.

In conclusion, a combination of IHC, immunocytochemistry and isolated cell culture using TRPV1 KO mice addressed the possibility that EGCs express TRPV1 and play a role in cell maturation. Further extensive studies are needed to validate this possibility.

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COMMENTS

Background

The transient receptor potential vanilloid 1 channel (TRPV1) is a non-selective cation channel that is activated by a wide variety of exogenous and endogenous physical and chemical stimuli, including heat, acidic condition and capsaicin. Enteric glial cells (EGCs) are one of major cell types comprising the enteric nervous system (ENS). This study explored TRPV1 expression in mouse EGCs.

Research frontiers

Although the presence of TRPV1 in astrocytes in the central nervous system has been reported, the presence in EGCs has not.

Innovations and breakthroughs

TRPV1-immunoreactive signal (TRPV1-IR) was detected in EGCs. The temporal retardation of postnatal maturation of EGCs in TRPV1 knockout mice was suggested.

Applications

The present results address possible involvement of TRPV1 in postnatal development/maturation of EGC. Dietary TRPV1 stimulation in the weaning period may affect postnatal ENS development. However, the expression and biological function of TRPV1 in EGC requires further evaluation.

Terminology

TRPV1 is a non-selective cation channel that is activated by a wide variety of exogenous and endogenous physical and chemical stimuli including heat, acidic condition and various pungent materials.

Peer-review

The authors are to be commended for the work in the manuscript entitled "TRPV1-immunoreactive signals in murine EGCs". This is an interesting paper highlighting the expression profiles of TRPV1 in murine EGCs.

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ORIGINAL ARTICLE

Basic Study

Anticancer effect of linalool *via* cancer-specific hydroxyl radical generation in human colon cancer

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Abstract

AIM

To investigate the anticancer mechanisms of the monoterpenoid alcohol linalool in human colon cancer cells.

METHODS

The cytotoxic effect of linalool on the human colon cancer cell lines and a human fibroblast cell line was examined using the WST-8 assay. The apoptosis-inducing effect of linalool was measured using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay and flow cytometry with Annexin V. Oxidative stress was investigated by staining for diphenyl-1-pyrenylphosphine, which is a cellular lipid peroxidation marker, and electron spin resonance spectroscopy. Sixteen SCID mice xenografted with human cancer cells were randomized into 3 groups for *in vivo* analysis: control and low-dose and high-dose linalool groups. The control group was administered tap water orally every 3 d. The linalool treatment groups were administered 100 or 200 μg/kg linalool solution orally for the same period. All mice were sacrificed under anesthesia 21 d after tumor inoculation, and tumors and organs were collected for immunohistochemistry using an anti-4-hydroxynonenal antibody. Tumor weights were measured and compared between groups.

RESULTS

Linalool induced apoptosis of cancer cells *in vitro*, following the cancer-specific induction of oxidative stress, which was measured based on spontaneous hydroxyl radical production and delayed lipid peroxidation. Mice in the high-dose linalool group exhibited a 55% reduction in mean xenograft tumor weight compared with mice in the control group ($P < 0.05$). In addition, tumor-specific lipid peroxidation was observed in the *in vivo* model.

CONCLUSION

Linalool exhibited an anticancer effect *via* cancer-specific oxidative stress, and this agent has potential for application in colon cancer therapy.

Key words: Colorectal cancer; Linalool; Oxidative stress; Electron spin resonance; Lipid peroxidation

Core tip: We elucidated the anticancer mechanism of the monoterpenoid alcohol, linalool, which induces apoptosis specifically in cancer cells *via* lipid peroxidation. Electron spin resonance (ESR) spectroscopy, which enables the real-time visualization of free radicals in live cells, revealed that oxidative stress developed immediately after treatment only in cancer cells. This study demonstrated that the natural compound linalool exerted an anticancer effect without causing serious side effects, and that the further utilization of ESR may support the application of linalool as a new and cost-effective cancer therapy.

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INTRODUCTION

Colorectal cancer is the fourth most common cause of cancer-related deaths globally, and the number of deaths has increased to approximately 700000 annually[1]. Chemotherapy is an effective treatments for colorectal cancer, but its side effects, such as hair loss, low blood counts, hand-foot syndrome, and neuropathy, may depress the patient's quality of life[2,3]. In addition, the current anticancer drugs are expensive[4]. Therefore, efforts are underway worldwide to identify new, effective, and inexpensive anticancer compounds with fewer side effects, and several types of natural compounds have recently been recognized as possible sources for anticancer drugs[5-9].

This study examined the anticancer effects of the monoterpenoid alcohol linalool, which is commonly used as a flavoring agent. Linalool is found abundantly in red wine, essential oil of lavender, and coriander fruits[10]. Several studies have reported the anticancer potential of linalool against solid tumor cell lines, such as gastric cancer, lung cancer, skin cancer[11], and hepatic cancer (HepG2)[12], as well as several leukemia cell lines[13]. Some of these studies reported that linalool also exerted an apoptotic effect[11,13], induced oxidative stress[12,14], and exhibited immunomodulation[15]. However, the mechanism by which linalool exerts its cytotoxic effect has not yet been elucidated[14]. We hypothesized that linalool's anticancer effects are mediated through the cancer-specific generation of hydroxyl radical followed by apoptosis. We investigated the cytotoxic effects of linalool in the human colon cancer cell line HCT 116 by analyzing the cell death mechanisms and measuring oxidative stress.

We focused on the detection of instant reactive oxygen species (ROS) production by using electron spin resonance (ESR) spectroscopy. ESR is a highly sensitive and the most definitive method for the detection of short-lived ROS using the spin-trapping technique, such as the hydroxyl radical, superoxide, and hydroperoxyl radical[16-18]. ESR was developed in the early 1970s, and it is often used in research of ischemia-reperfusion injury[19-21] and oxidative stress after exercise[22]. The method is not commonly used in cancer biology studies, but it has potential for

wide application in cancer screening and therapeutic evaluation in the near future, because it is becoming evident that both the ROS levels and redox signaling can affect the phenotypic profile of cancer cells and their responsiveness to therapeutic interventions[23,24].

MATERIALS AND METHODS

Drugs

Linalool (97% pure; Sigma Aldrich, St. Louis, MO, USA), diphenyl-1-pyrenylphosphine(DPPP) (Dojindo, Kumamoto, Japan), 5,5-dimethyl-1-pyrroline-N-oxide(DMPO) (Radical Research Inc., Tokyo, Japan), dimethyl sulfoxide (DMSO) (Wako, Osaka, Japan), and Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) were purchased.

Animals and xenograft tumors

Six-week-old male severe combined immune deficiency (SCID) mice (Clea, Tokyo, Japan) were maintained in plastic cages in a temperature-controlled room on a 12-h light/dark cycle with free access to water and a standard pellet diet throughout the experiment. After an acclimation period of 7 d, the solid tumor was developed by the subcutaneous inoculation of 1×10^6 HCT 116 cells on the right flank of each mouse. The mice were divided into the following three groups: control group (n = 5), mice treated with saline; low-dose linalool group (n = 5), mice treated with 100 mg/kg linalool; and high-dose linalool group, mice treated with 200 mg/kg linalool (n = 6). Seven days after tumor injection, saline, low-dose linalool, and high-dose linalool were administered orally *via* gavage every 3 d in a volume of 25 μL. All the animals were sacrificed 21 d after tumor inoculation. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were detected. All animal experiments were performed in a humane manner after approval from the Institutional University Experiment Committee of the University of Tsukuba (protocol number: 15-254).

Cell culture and viability

Human colon cancer cell lines, *e.g*., HCT 116, and a human fibroblast cell line CCD18Co were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and subdivided into multiple tubes for stocking in liquid nitrogen. HCT 116: a human colon cancer cell line that is commonly used to study cancer invasion and tumorigenesis (passage 4), WiDr: colon adenocarcinoma cell line established from a 78 year old female (passage 6), RKO: a poorly differentiated colon carcinoma cell line (passage 4), HCT-15: a quasidiploid human colon cancer cell line (passage 6), SW480: established from Dukes' type B, colorectal adenocarcinoma cells (passage 5), and CCD18Co: a human fibroblast cell line used as a substitute for normal cells (passage 6), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with inactivated 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured in a 5% CO_2 cell culture incubator at 37 ℃.

Cell viability was examined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Cancer cells ware seeded in a 96-well dish at 3000 cells/well, incubated overnight, and incubated with different concentrations of linalool for 24 h. The medium was replaced with the medium containing 10% WST-8, and the cells were incubated further for 2 h. The absorbance of each well at 450 nm was measured by a SUNRISE Rainbow microplate reader (TECAN, Groedig, Austria).

Apoptosis assays

The In Situ Cell Death Detection kit(Roche Diagnostics, Basel, Switzerland) was used to detect apoptosis in the cell culture. HCT 116 cells were seeded at 3000 cells/well onto Lab-Tek II Chamber Slides (Nalge Nunc International, Tokyo, Japan) and incubated overnight. Cells were incubated with linalool (0, 100 μmol/L) or 50 nM staurosporine as a positive control for 24 h. Cells were incubated with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) reaction mixture according to the manufacturer's instructions.

A flow cytometer (Muse Cell Analyzer) and Muse Annexin V & Dead Cell Kit (Merck Millipore, Darmstadt, Germany) were used for quantitative analyses of live, early and late apoptosis, and cell death in suspension cell lines. HCT 116 cells were seeded at a density of 1×10^5 cells per dish (diameter, 6 cm) and incubated overnight. Cells were incubated with different concentrations of linalool (0, 10, 100, 1000 μmol/L) and 50 nM staurosporine as the positive control for 24 h. Cells were prepared with Annexin V and Dead Cell reagent according to the manufacturer's instructions, and the samples were evaluated using the MUSE analyzer with the appropriate thresholds for cell size and apoptotic profile.

Lipid peroxidation test using the diphenyl-1-pyrenylphosphine assay

HCT 116 cells and CCD18Co cells were seeded at a density of 3000 cells/well each onto Lab-Tek Chambered Borosilicate Coverglass Slides (Nalge Nunc International, Tokyo, Japan) and incubated overnight. CCD18Co cells were used as a normal cell control. diphenyl-1-pyrenylphosphine (DPPP) (5 mmol/L) was dissolved in DMSO, and the solution was diluted using DMEM without Phenol red or FBS. The final concentration was adjusted to 50 μmol/L. A volume of 500 μL of the solution was added to each well and incubated for 10 min. Cells were incubated with different concentrations of linalool for 2 h. The oxidation of DPPP was monitored using KEYENCE BZ-X700 (Keyence, Osaka, Japan). Wavelengths of

excitation and emission were set at 352 nm and 380 nm, respectively.

Electron spin resonance spectroscopy

HCT 116 cells were cultured on rectangular cover slides and incubated overnight. Cells were incubated for 15 min with different concentrations of linalool (0, 250, 500, 1000 μmol/L) and were immersed in a culture medium with 5 mmol/L respiratory substrates (succinate, glutamate, and malate), 5 mmol/L nicotinamide adenine dinucleotide (NADH), and 10 mmol/L DMPO. Immersed-slide glasses were placed on the tissue glass and measured using the EPR apparatus for 10 min, 30 min, 1 h, and 2 h after treatment. All spectra were recorded using a JEOL-TE X-band spectrometer (JEOL Ltd., Tokyo, Japan) and analyzed using Win-Rad Radical Analyzer System (Radical Research, Inc., Tokyo, Japan).

Histological and immunohistochemical assays

Immunohistochemistry for 4-HNE was performed in all samples as follows. Paraffin was removed with xylene, and endogenous peroxidase was blocked in tissues using 3% of H_2O_2 in methanol. All tissue sections were pretreated in citrate buffer (pH 6.0) for 10 min at 100 ℃ in a microwave oven. Nonspecific binding sites were blocked using 10% BSA before incubation with the primary antibody. Slides were washed, incubated with biotinylated secondary IgG for 30 min and exposed to a biotin-peroxidase complex.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. Significant probability (*P* value) was calculated using ANOVA followed by Dunnett's test. The results are expressed as the mean ± SD, and $P < 0.05$ was considered statistically significant.

The statistical methods of this study were reviewed by Isao Muraki, MD, PhD, an epidemiologist in the Department of Cardiovascular Disease Prevention, Osaka Center for Cancer and Cardiovascular Disease Prevention.

RESULTS

Linalool induces apoptosis in human colon cancer cells

Phase contrast microscopy was used to observe the morphologic changes of HCT 116 cell line, and the WST assay was performed for each cell line to evaluate cytotoxicity after exposure to linalool. Cancer cells exhibited rapid proliferation under control conditions (Figure 1A). In contrast, cancer cells stopped proliferating after treatment with 250 μmol/L linalool and exhibited fragmentation of chromatin, bleb formation on the cell surface, and shrinkage, which are representative of apoptosis (Figure 1B). We observed a dose-dependent increase in the cytotoxic effect of linalool in all cancer cell lines and fibroblast cell lines, but the cell viability of CCD18Co was suggested to be higher than that of HCT 116 at 100-500 μmol/L (Figure 1C). Micrographs of HCT 116 cells treated with different linalool concentrations for 24 h revealed shrinkage of cells and tightly packed organelles at concentrations over 100 μmol/L (data not shown).

TUNEL staining was performed using HCT 116 cells to clarify the mechanism of linalool cytotoxicity. The cells were divided into the following three groups: control, linalool, and staurosporine groups. The corresponding percentages of TUNEL-positive cells were 0.7% ± 0.7% (Figure 2A), 10.5% ± 7.7% (with 100 μmol/L linalool treatment; Figure 2B), and 25.1% ± 11.2% (with 100 μmol/L staurosporine treatment; Figure 2C). The percentage of apoptotic cells in each group is shown as a column graph with error bars (Figure 2D).

The Annexin V assay was performed for a further quantitative analysis of live cells and cells showing early and late apoptosis, and cell death. Figure 2E and 2F shows the percentage of gated cells in each quadrant, and the data revealed a dose-dependent increase in cells in early and late apoptosis phases.

These results clearly demonstrated that linalool induced apoptosis of human colon cancer cells in a dose-dependent manner *in vitro*.

Linalool inhibits the growth of human xenografted tumor mouse model without side effects

A human cancer xenografted mouse model was established, and linalool was administered orally to investigate whether linalool exhibited an anticancer effect *in vivo*. Treatment with linalool caused a decrease in the size and weight of subcutaneous tumors (Figure 3A). And the difference in the resected tumor weight between the control group and the high-dose (200 mg/kg) linalool treatment group was significant (Figure 3B). No changes in the mean body weight and serum AST and ALT levels among the three groups were detected (Figure 3C). Skin damage, hair loss, intestinal bleeding, and other abnormal symptoms were not observed in any of the three groups. These results demonstrated that linalool exhibited an antiproliferation effect on transplanted human cancer cells without significant weight loss or damage to organs.

Linalool induces cancer-specific oxidative stress and apoptosis

An oxidative stress assay was performed to further confirm the mechanism of the apoptotic effect of linalool. ESR spectroscopy was performed in HCT 116 and CCD18Co cells using DMPO as a spin-trapping reagent. The signal intensity of DMPO reflects linalool-induced hydroxyl radical production. Cancer-specific signal intensity gain was observed only for 500 μmol/L linalool 30 min after treatment (Figure 4A), which was diminished at an earlier time point (10 min) and at later time points (1 and 2 h). The signal intensity of

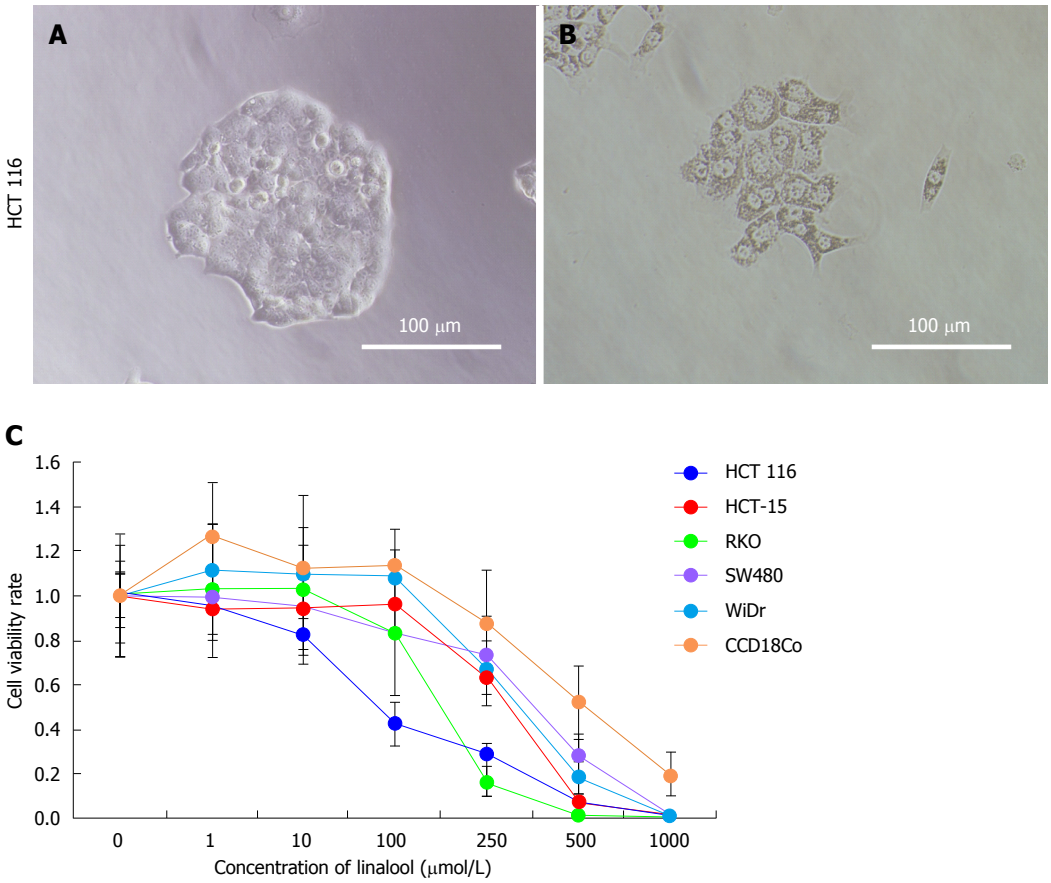

Figure 1 Anticancer effect of linalool. A: HCT 116 cells in the control group showing rapid proliferation; B: HCT 116 cells treated with 250 μmol/L linalool for 24 h showed shrinkage of cells and tightly packed organelles; C: A dose-dependent increase in the cytotoxic effect of linalool was observed in all cancer cell lines. Values are expressed as mean ± SD (n = 3).

control is shown in (Figure 4B). There was no gain in signal intensity for CCD18Co cells at any time point (data not shown). The DPPP assay was performed to detect lipid peroxidation as green fluorescence on the cell surface. Linalool induced HCT 116 cell membrane lipid peroxidation(Figure 4C), whereas the signal was not observed without linalool(Figure 4D). CCD18Co also showed no lipid peroxidation by linalool (data not shown).

Histological analyses of mouse tumor samples demonstrated a reduction of cancer cells and a relatively large area of necrotizing tissue inside the tumor. Immunohistochemistry for 4-HNE, which is a marker of oxidative stress due to increased lipid peroxidation, revealed an accumulation of 4-HNE in tumors from the high-dose linalool group compared to tumors from the control group. However, no significant difference in 4-HNE accumulation in stomach or liver tissues was observed between any of the groups (Figure 5).

These results revealed that linalool induced cancer-specific oxidative stress that was first induced in the form of hydroxyl radical production, followed by lipid peroxidation, and exhibited a selective anticancer apoptotic effect.

DISCUSSION

In this study, we have demonstrated for the first time that the rapid induction of ROS by linalool as a trigger of subsequent apoptotic cascade *via* lipid peroxidation with living-cellular ESR technique. We also revealed that this mechanism was cancer specific and significant weight loss or damage to normal organs were not observed in the human cancer xenografted mice with oral administration of linalool. These results raise the possibility that linalool is applicable as a new resource for cancer therapy.

The anticancer activity of some essential oils from various plants has received substantial attention in recent years because these essential oils are likely to serve as new sources of chemotherapeutic drugs[25]. Existing chemotherapeutic agents exert cytotoxic effect not only on cancer cells but also on normal cells, and various side effects are observed. In contrast, some essential oils exhibit maximum efficacy against cancer cells and cause minimal toxic effects in normal cells[26]. The elucidation of the mechanism by which these essential oils exert the anticancer effect possibly contribute to the improvement in survival rate and quality of life (QOL) of cancer patients. Monoterpenes

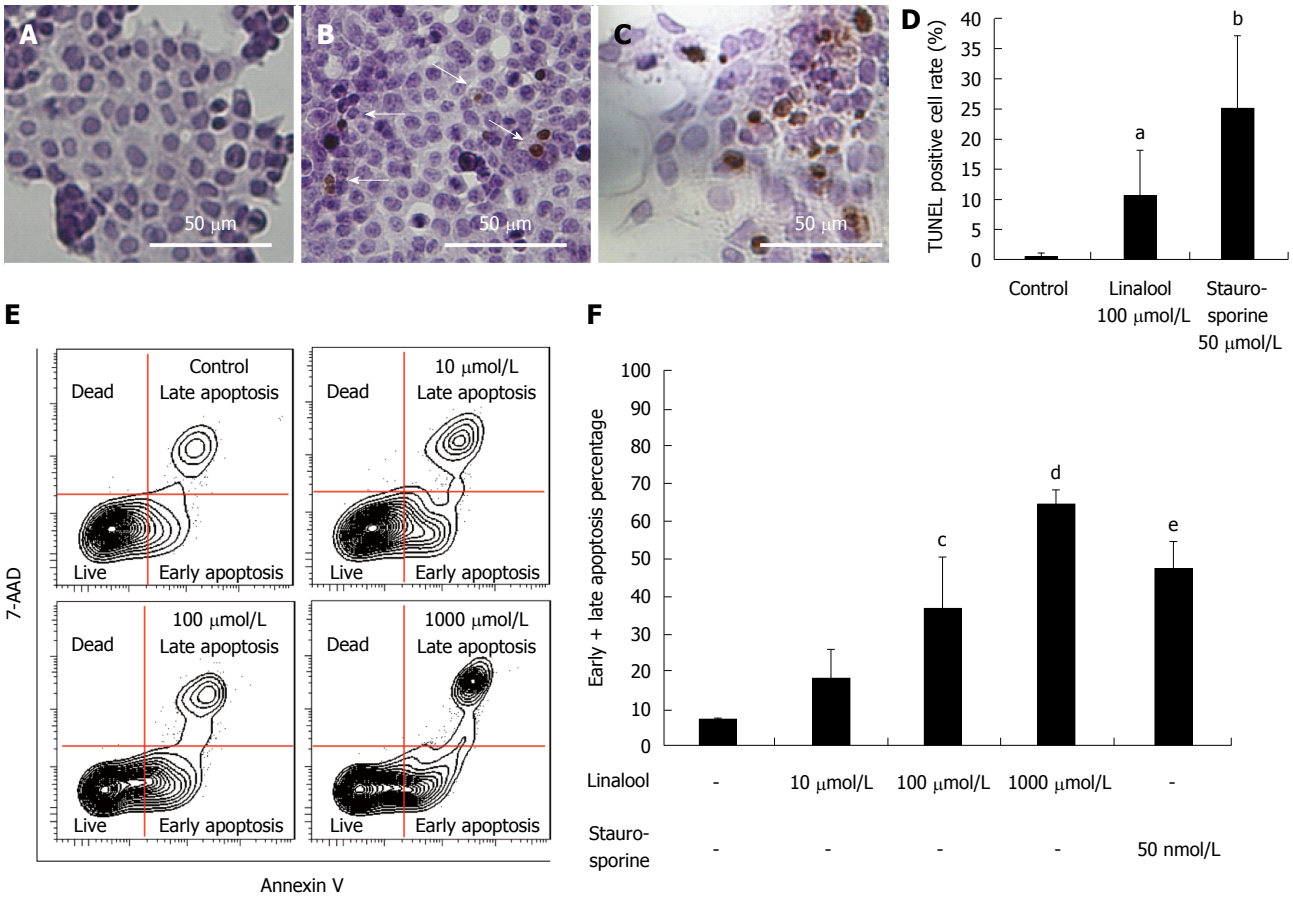

Figure 2 Anticancer mechanism of linalool. A: TUNEL staining of HCT 116 cells in the control group; B: Staining of HCT 116 cells treated with 100 μmol/L linalool; C: Staining of HCT 116 cells treated with 50 nmol/L staurosporine. The corresponding percentage of TUNEL-positive cells (white arrow) was 0.7% ± 0.7% (A), 10.5% ± 7.7% (B), and 25.1% ± 11.2% (C); D: The percentage of apoptotic cells in each group (mean ± SD, n = 10). $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* 100 μmol/L linalool group; E: Flow cytometry using Annexin V for the detection of live cells, cells showing early and late apoptosis, and cell death; F: Percentages of early and late apoptosis fraction. $^{c}P < 0.05$ *vs* control group; $^{d}P < 0.01$ *vs* control group; $^{e}P < 0.05$ *vs* control group; one-way ANOVA followed by Dunnett's test, n = 3.

are non-nutritive dietary component found in the essential oils of citrus fruits, mints and herbs and belong to a group of chemical compounds named "terpenes", representing a group of natural compounds whose basic structure consists of two linked isoprene units, which are formed by a 5-carbon base each[27]. Linalool is an acyclic monoterpenoid alcohol with a chemical formula of $C_{10}H_{18}O$ and molecular mass of 154.25 g/mol, and has widely been used for its fragrance and odorant qualities in cosmetics, soaps, and perfumes. It is an inexpensive, familiar, and well-known chemical with little toxicity for a human body. According to the aforementioned references, there are three phenomena as anticancer activities of linalool: apoptotic effect[11,13], induction of oxidative stress[12,14], and immunomodulation[15]. However, its underlying mechanism and the reason why linalool exerts cancer selective cytotoxicity have not been confirmed. Therefore, we focused on the detection of instant reactive oxygen species (ROS) production by using ESR spectroscopy.

Free electrons of ROS align with their low energy status (βspin) or high energy status (αspin) in an external magnetic field. A transition between low- and high-energy states may occur when sufficient microwave energy is absorbed[28]. An ESR spectrum is obtained and recorded by observing variations in magnetic field strength at a fixed microwave frequency. ROS are generally present at low concentrations and very reactive, and the use of spin-trapping reagents solves these problems. The ESR spin-trapping technique involves the use of chemical species called spin traps, which react with short-lived free radicals to form relatively stable adducts with a half-life that is adequate to observe the ESR spectrum[29]. This study used DMPO as a spin trap to specifically detect hydroxyl radicals with a signal intensity of 1:2:2:1[29]. Furthermore, more than 90% of intracellular ROS are generated from mitochondria, and these ROS peroxidize lipids at the same location and produce HNE around the mitochondria, which leads to apoptosis[30]. ROS was detected almost immediately and directly after treatment by using an ESR system. The findings of our *in vitro* experiments following ESR signal detection are consistent with the aforementioned findings, which means that ROS generation, as a sign of apoptosis of cancer cells. The result of our study indicates that detection of ROS by

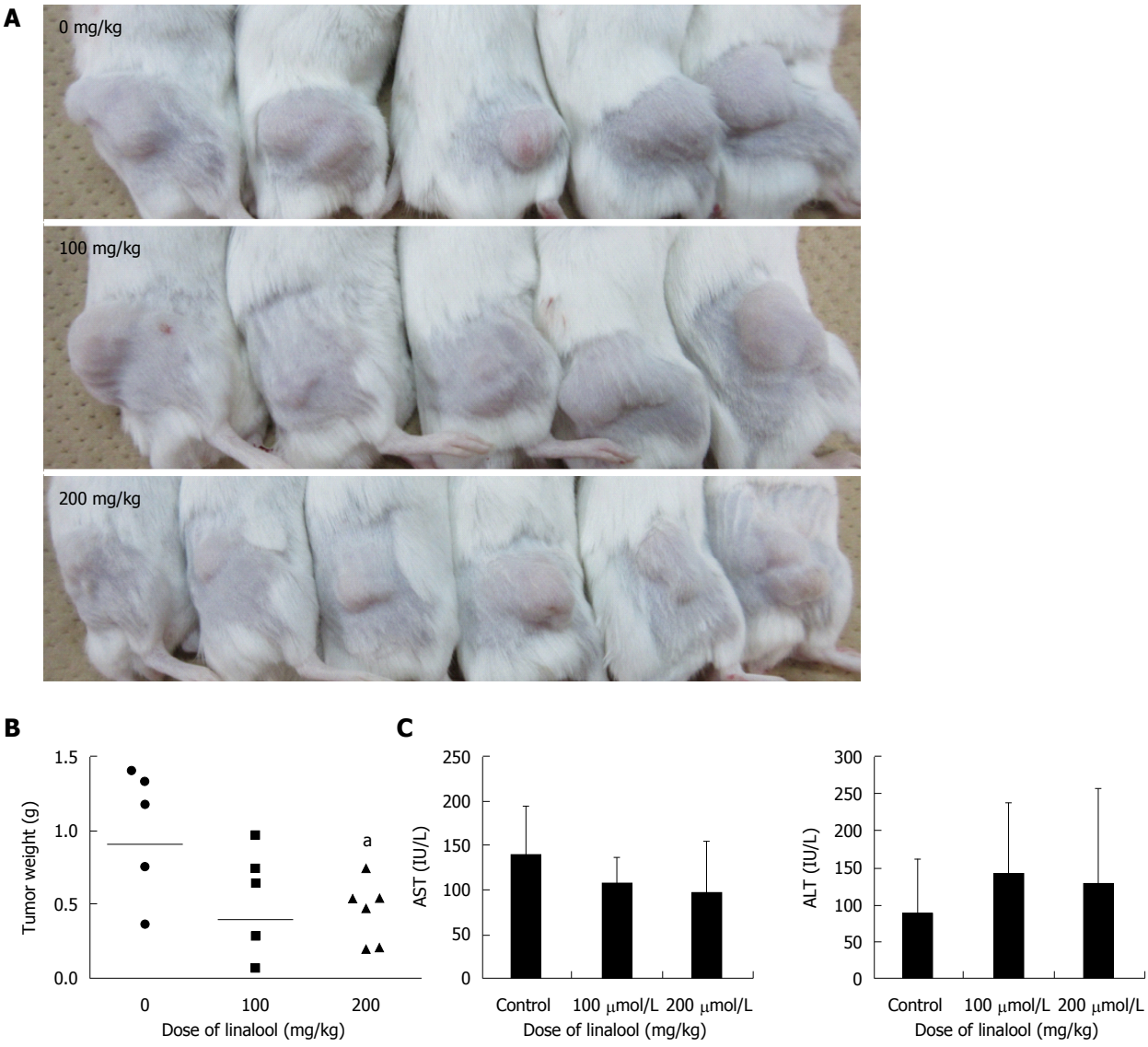

Figure 3 Changes in mouse tumor size and weight after drug administration. A: The pictures show the size of the tumors in each group. Upper: control group. Middle: 100 mg/kg linalool group. Lower: 200 mg/kg linalool group. As concentration of linalool increased, the xenografted tumor size decreased macroscopically; B: Tumor weight after removal showed a significant decrease in the group of 200 mg/kg administration, compared to that of control group [expressed as geometric mean, n = 5 (control), 5 (100 mg/kg linalool), 6 (200 mg/kg linalool)], $^{a}P < 0.05$ *vs* control group; one-way ANOVA followed by Dunnett's test); C: No significant change was detected in serum AST and ALT level in each group; values are expressed as mean ± SD, n = 5 (control), 5 (100 mg/kg linalool), 6 (200 mg/kg linalool).

ESR is applicable to the screening and therapeutic evaluation of anticancer agents.

The results obtained from this study showed that linalool has distinct roles in lipid peroxidation in cancer and other tissues such as the stomach and the liver. It has been described that such a cancer selective effect probably depend on the redox state of the cell[31]. The cancer cellular concentrations of ROS used to be higher than that of normal cells because the cancer cellular metabolism is completely different: it is well known as the Warburg effect[32]. Under such condition, cancer cells should have a higher ability for ROS regulation as its survival strategy[33,34]. This is a reason why cancer cells can endure the oxidative stress environments. In addition, one recent study reported an inhibitory effect of linalool on mitochondrial complexes I and II, the electron transport, followed by mitochondrial derangements and cell death in HepG2 cells[12]. Thus, the difference of the redox, metabolic, and mitochondrial states between the cancer and normal organ tissues might be responsible for linalool's selective oxidative stress induction and selective cytotoxicity to cancer cells. Although we focused on the instant generation of ROS using ESR, which produced complementary findings, further studies are required to establish the strategy to produce a state of selective oxidative stress in cancer cells.

In conclusion, we demonstrated that linalool induced apoptosis of human colon cancer cells *via* cancer-specific oxidative stress, which was detectable as an ESR signal. The further utilization of ESR can reveal novel uses for natural compounds, such as linalool, as

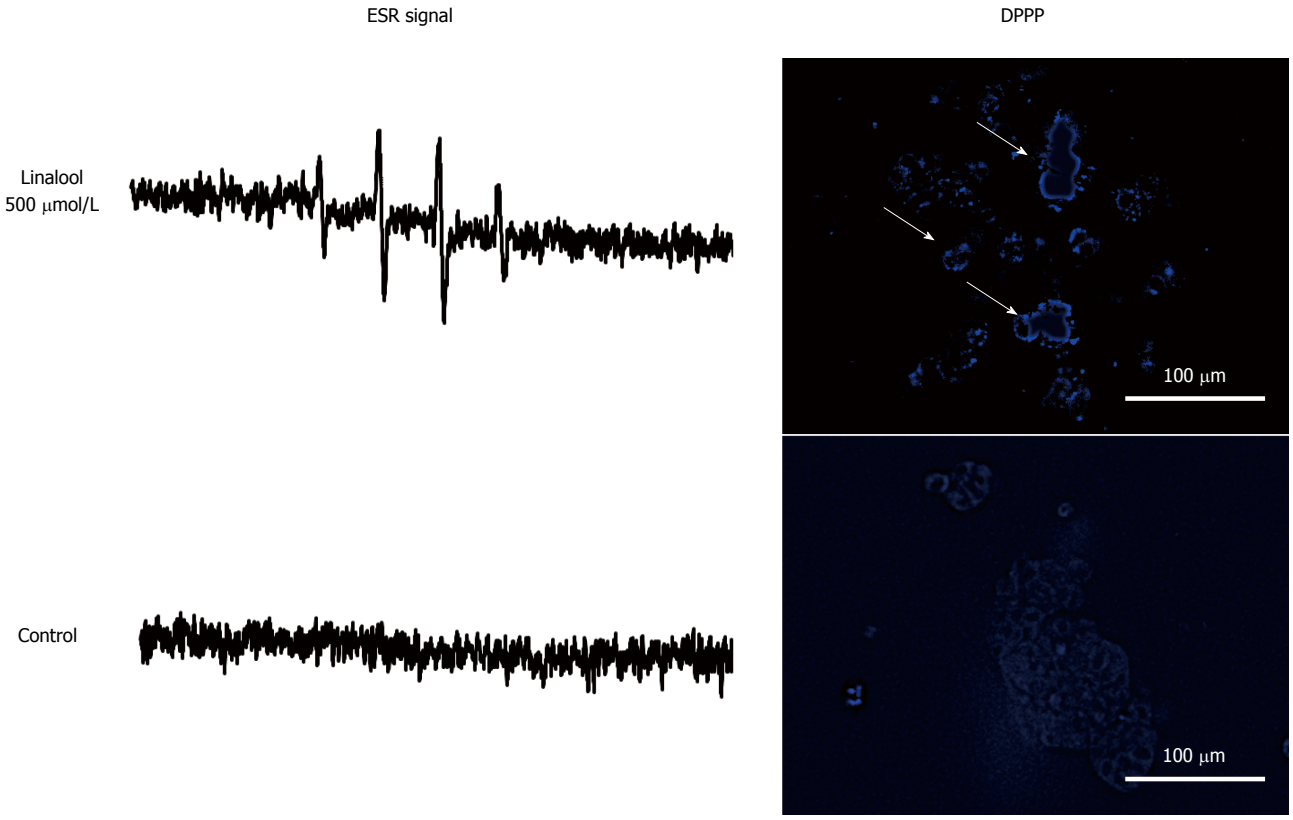

Figure 4 Oxidative stress assay *in vitro*. A: Electron spin resonance (ESR) spectroscopy performed 30 min after linalool treatment. The signal intensity of 1:2:2:1 suggests generation of hydroxyl radical, the strongest reactive oxygen species (ROS); B: The signal of HCT 116 without linalool at the same time period; C: Diphenyl-1-pyrenylphosphine (DPPP) assay performed to detect lipid peroxidation showed that linalool induced cell membrane lipid peroxidation as green fluorescent on the cell surface(white arrow); D: The cell membrane lipid peroxidation was not observed in without linalool treatment group.

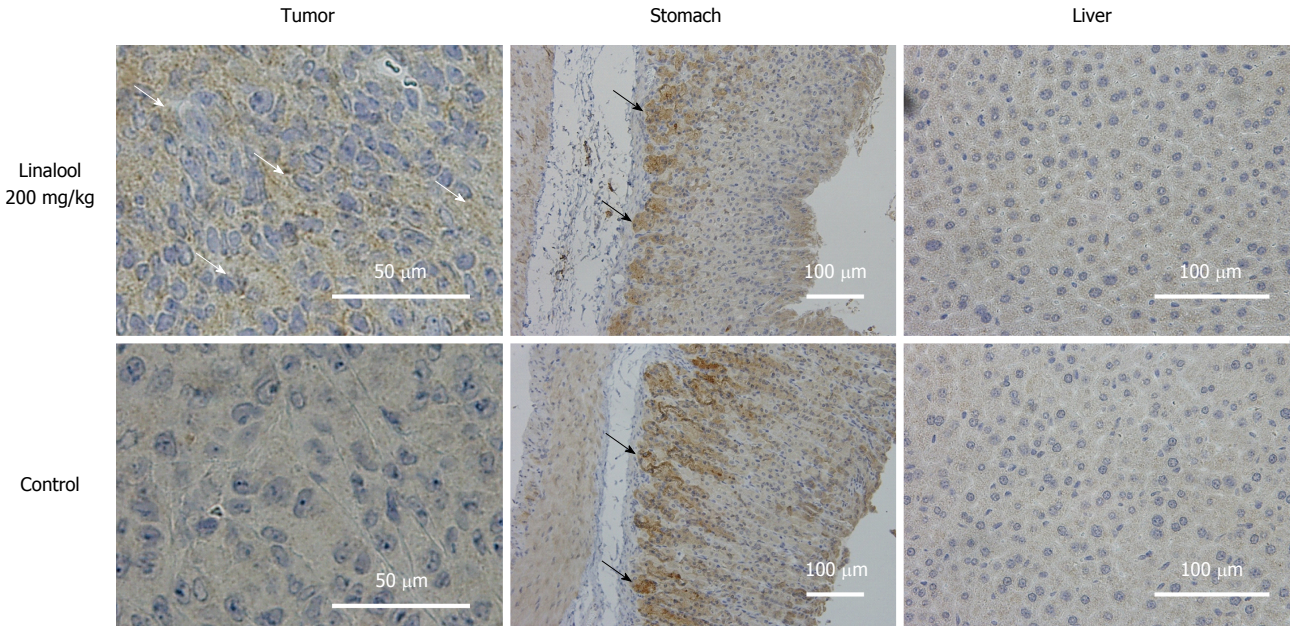

Figure 5 Oxidative stress assay *in vivo*. Although accumulation of 4-hydroxynonenal (4-HNE) was observed in the tumors from the high-dose linalool group, it was not observed in stomach or liver tissues, except for the physiological accumulation (black arrow) of 4-HNE near the basal membrane of the stomach.

new, cost-effective cancer therapies.

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COMMENTS

Background

Colorectal cancer is a major public health problem, and its mortality is very high globally. Chemotherapy is the most effective treatment for colorectal cancer, but the problems of side effects and high costs remain unresolved.

Research frontiers

There is a growing interest in the search for the cancer preventive or therapeutic potential of natural compounds, which are not expensive and exhibit few side effects. Linalool is a monoterpenoid alcohol that is present abundantly in red wine and coriander and exhibits anticancer effects in some types of human cancer. However, the mechanisms of this effect are not clear.

Innovations and breakthroughs

The authors focused on the detection of instant reactive oxygen species (ROS) production using electron spin resonance (ESR) spectroscopy, which is a highly sensitive and definitive method for the detection of short-lived ROS. We revealed that the first sign of apoptosis in cancer cells induced by linalool may be detected almost immediately and directly after treatment by using an ESR system. This result is an important finding for the screening and therapeutic evaluation of new anticancer agents.

Applications

This study was designed to evaluate the anticancer mechanisms of linalool on human colon cancer cell lines for the development of new effective anticancer strategies.

Terminology

Linalool is an acyclic monoterpenoid alcohol with a chemical formula of $C_{10}H_{18}O$ and molecular mass of 154.25 g/mol. Approximately 12000 T of linalool is produced for the industrial use annually, and its natural biosynthesis through plants, primarily herbs, trees and fruits, is higher by dimension. More than 95% of synthetic linalool is used for its fragrance and odorant qualities in cosmetics, soaps, and perfumes, and only approximately 1% is added to food and beverages for aroma and flavoring. Linalool is an inexpensive, familiar, and well-known chemical with great prospects for medical application.

Peer-review

Article "Anticancer activity of linalool *via* cancer-specific hydroxyl radical generation in human colon cancer", provides significant and interesting results regarding the anticancer mechanism of linalool by inducing apoptosis specifically in cancer cells *via* lipid peroxidation.

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ORIGINAL ARTICLE

Basic Study

Naringenin protects against isoniazid- and rifampicin-induced apoptosis in hepatic injury

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Abstract

AIM

To explore the protective effects and mechanisms of naringenin (NRG) on hepatic injury induced by isoniazid (INH) and rifampicin (RIF).

METHODS

Male mice were randomly divided into four groups and treated for 14 d as follows: normal control group was administered intragastrically with normal saline solution alone; model group was administered intragastrically with INH (100 mg/kg) and RIF (100 mg/kg); low- and high-dosage NRG pretreatment groups were administered intragastrically with different doses of NRG (50 or 100 mg/kg) 2 h before INH and RIF challenge. Mice were killed 16 h after the last dose of drug treatment to determine activity of serum transaminases. Oxidative stress was evaluated by measuring hepatic glutathione (GSH) and superoxide dismutase (SOD) and malondialdehyde (MDA) levels. Histopathological changes in hepatic tissue were observed under the optical microscope. Hepatocyte apoptosis was measured

by TUNEL assay and caspase-3 activation. Expression of Bcl-2 and Bax in liver was determined by western blot.

RESULTS

Both low- and high-dosage NRG pretreatment obviously alleviated serum levels of alanine aminotransferase and aspartate aminotransferase, liver index, hepatic MDA content, and increased hepatic GSH content and SOD activity compared with the INH and RIF-treated group (44.71 ± 8.15 U/L, 38.22 ± 6.64 U/L *vs* 58.15 ± 10.54 U/L; 98.36 ± 14.78 U/L, 92.41 ± 13.59 U/L *vs* 133.05 ± 19.36 U/L; 5.34% ± 0.26%, 4.93% ± 0.25% *vs* 5.71% ± 0.28%; 2.76 ± 0.67 nmol/mgprot, 2.64 ± 0.64 nmol/mgprot *vs* 4.49 ± 1.12 nmol/mgprot; 5.91 ± 1.31 mg/gprot, 6.42 ± 1.42 mg/gprot *vs* 3.11 ± 0.73 mg/gprot; 137.31 ± 24.62 U/mgprot, 148.83 ± 26.75 U/mgprot *vs* 102.34 ± 19.22 U/mgprot; all $P < 0.01$ or 0.05). Histopathological evaluation showed obvious necrosis and inflammatory cell infiltration in liver of mice administered INH and RIF; however, mice pretreated with NRG showed minor hepatic injury. In addition, INH and RIF resulted in hepatocyte apoptosis, and NRG pretreatment dramatically suppressed INH- and RIF-induced hepatocytes apoptosis. Furthermore, NRG-mediated anti-apoptotic effects seemed to be in connection with its regulation of Bax and Bcl-2 protein expression in hepatic tissue.

CONCLUSION

NRG might attenuate INH- and RIF-induced hepatic injury *via* suppression of oxidative stress and hepatocyte apoptosis.

Key words: Naringenin; Isoniazid; Rifampicin; Oxidative stress; Apoptosis; Hepatic injury

Core tip: Anti-tuberculosis drugs can cause drug-induced liver injury through a series of complex mechanisms. The main purposes of the study were to explore the effects of naringenin (NRG) on liver injury induced by isoniazid (INH) and rifampicin (RIF). Mice were administered intragastrically with NRG before INH and RIF challenge. The findings revealed that NRG protects against INH- and RIF-induced oxidative stress and hepatocyte apoptosis.

Wang C, Fan RQ, Zhang YX, Nie H, Li K. Naringenin protects against isoniazid- and rifampicin-induced apoptosis in hepatic injury. *World J Gastroenterol* 2016; 22(44): 9775-9783 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9775.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9775

INTRODUCTION

Tuberculosis (TB) is a deadly communicable disease caused by mycobacteria, and it remains a serious global public health issue. In 2014, about 9.6 million people fell ill with TB and 1.5 million died from the disease[1]. The TB treatment recommended by the World Health Organization is a regimen of isoniazid (INH), rifampicin (RIF), pyrazinamide, and ethambutol for 2 mo in the initiation stage, followed by 4 mo of INH and RIF in the continuation stage. Unfortunately, the two most common anti-TB drugs, INH and RIF, can cause drug-induced hepatic injury through a series of complex mechanisms. Peroxidation of endogenous lipids has been shown to be the dominant factor in the hepatotoxic action of INH and RIF[2]. Anti-TB drug-mediated oxidative injury is usually ascribed to formation of highly reactive oxygen species, which act as stimulators of lipid peroxidation and damage the cell membrane[3]. Oxidative stress in the hepatocyte results in altered mitochondrial permeability and induces apoptosis[4]. Therefore, antioxidants could serve as possible drugs against INH- and RIF-induced hepatic toxic injury[5,6].

In recent years, natural plant extracts have been extensively regarded as a rich resource for drug development. Naringenin (NRG) is a type of natural flavonoid compound, which widely exists in grapefruit, bitter orange and other plants. NRG has wide range of biological and pharmacological activities, such as anti-inflammatory[7], anti-mutagenic[8], anti-atherogenic[9], anti-oxidant[10] and anti-cancer[11]. Related studies have shown that NRG attenuates liver injury induced by lead[12], acetaminophen[13], cholesterol overdose[14] and carbon tetrachloride (CCl_4) intoxication[15]. It was suggested that NRG can exert hepatoprotective effects and may has therapeutic value for various liver diseases.

Therefore, this study was conducted to investigate the potent protective effects of NRG on INH- and RIF-induced hepatic injury in mice. In addition, we discussed the underlying mechanisms of its therapeutic action.

MATERIALS AND METHODS

Reagents

NRG, INH and RIF were purchased from Sigma Aldrich (St. Louis, MO, United States). Kits to measure aspartate aminotransferase (AST), alanine aminotransferase (ALT), superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA) were purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China). TUNEL detection kit was purchased from Boster Biological Engineering Co., Ltd (Wuhan, China). Rabbit anti-mouse cleaved caspase-3 monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, United States). Rabbit anti-mouse Bax, Bcl-2 and β-actin polyclonal antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, United States).

Animals

Forty male specific pathogen-free BALB/c mice, weighing 20-22 g, were purchased from the Central Animal Facility of Wuhan University (Wuhan, China). All mice were housed in an environmentally controlled room (25 ± 2 ℃, 55% ± 5% humidity) with a 12 h light and dark cycle and had free access to laboratory standard food and water. All experiments received approval by the Yangtze University's institutional laboratory animal care and use committee.

Drug administration

After an acclimatization period of 1 wk, animals were randomly divided into four equal groups, each consisting of 10 mice, and treated for 14 d as follows: normal control group was administered intragastrically with normal saline solution (NSS) alone; model group was administered intragastrically with INH (100 mg/kg) and RIF (100 mg/kg); low-dosage and high-dosage NRG pretreatment groups were administered intragastrically with different doses of NRG (50 or 100 mg/kg) 2 h before INH and RIF challenge. NRG was dissolved in 5% carboxyl methyl cellulose and then diluted with NSS to the required concentration for oral administration. Mice were killed 16 h after the last dose of drug treatment to collect serum and liver tissue samples.

Biochemical analysis

The liver index was calculated according to the formula: liver index (%) = mouse liver weight (g)/mouse weight (g) × 100%. The serum biochemical parameters, such as ALT and AST, were measured by absorbance photometry using commercial automated chemistry analyzers.

GSH level was assayed in the liver tissue according to the method of Griffith[16] and concentration was expressed as mg/g protein. SOD activity was assayed in the liver tissue through nitroblue tetrazolium coloration according to the manufacture's protocols and was expressed as U/mg protein. Lipid peroxidation was assayed by measuring MDA in the liver tissue according to the method of Wills[17], and MDA content was expressed as nmol/mg protein.

Histopathology

Small pieces of liver were fixed in 10% neutral buffered formalin and then embedded into paraffin. Paraffin-embedded sections at 6 μm and stained with hematoxylin and eosin, and then analyzed under the optical microscope for histopathological evaluation.

TUNEL staining

The sections were stained for detection of apoptosis in live cells using a commercially available TUNEL kit and following the manufacturer's instructions. The ratios of apoptosis were analyzed under the optical microscope by counting TUNEL positive cells in six randomly selected areas.

Immunohistochemistry

The sections were dewaxed in xylene and rehydrated in descending graded alcohols, followed by incubation in 3% hydrogen peroxide for 15 min to neutralize endogenous peroxidase activity, and then microwave antigen retrieval for 15 min in citric acid buffer. Nonspecific binding was blocked with 10% normal goat serum. Subsequently, the sections were incubated overnight with a rabbit anti-mouse cleaved caspase-3 monoclonal antibody at 4 ℃, and then the goat anti-rabbit secondary antibody (Zhongshan Golden Bridge Biotechnology Company, China) was incubated at room temperature for 30 min. Finally, the sections were treated with diaminobenzidine working solution, and then counterstained with hematoxylin and covered with cover slips.

Western blotting

Proteins were extracted from liver tissue using the Bullet Blender homogenizer (Next Advance Inc., Averill Park, NY, United States) in RIPA lysis buffer (Beyotime, Shanghai, China), according to the manufacturer's protocols. Equal amounts of proteins were subjected to electrophoresis on 10% SDS-polyacrylamide gel electrophoresis, and then the separated protein bands were transferred onto polyvinylidene difluoride membranes (Hybond, Escondido, CA, United States). Next, the membranes were incubated respectively with rabbit anti-mouse β-actin, Bax or Bcl-2 polyclonal antibodies overnight at 4 ℃. After incubation with the goat anti-rabbit secondary antibody, proteins were detected by enhanced chemiluminescence detection kit (Pierce, Rockford, IL, United States). The densitometric analysis of the protein bands was carried out with Image J software.

Statistical analysis

All data were expressed as mean ± SEM. Statistical differences were analyzed by Student's *t* test or one-way ANOVA. The analysis was conducted using SPSS 17.0 software. *P* values below 0.05 were considered statistically significant.

RESULTS

NRG pretreatment alleviates INH- and RIF-induced liver injury

To investigate the protective effects of NRG against INH and RIF, levels of serum transaminases were analyzed. As shown in Figure 1A, administration of INH and RIF led to a significant increase in serum ALT and AST levels, whereas NRG pretreatment obviously alleviated INH- and RIF-induced elevation of ALT and AST (44.71 ± 8.15 U/L, 38.22 ± 6.64 U/L *vs* 58.15 ± 10.54 U/L; 98.36 ± 14.78 U/L, 92.41 ± 13.59 U/L *vs* 133.05 ± 19.36 U/L; all $P < 0.01$). Next, as shown in Figure 1B, administration of INH and RIF led to a significant increase in the liver index, while NRG pretreatment obviously attenuated INH- and

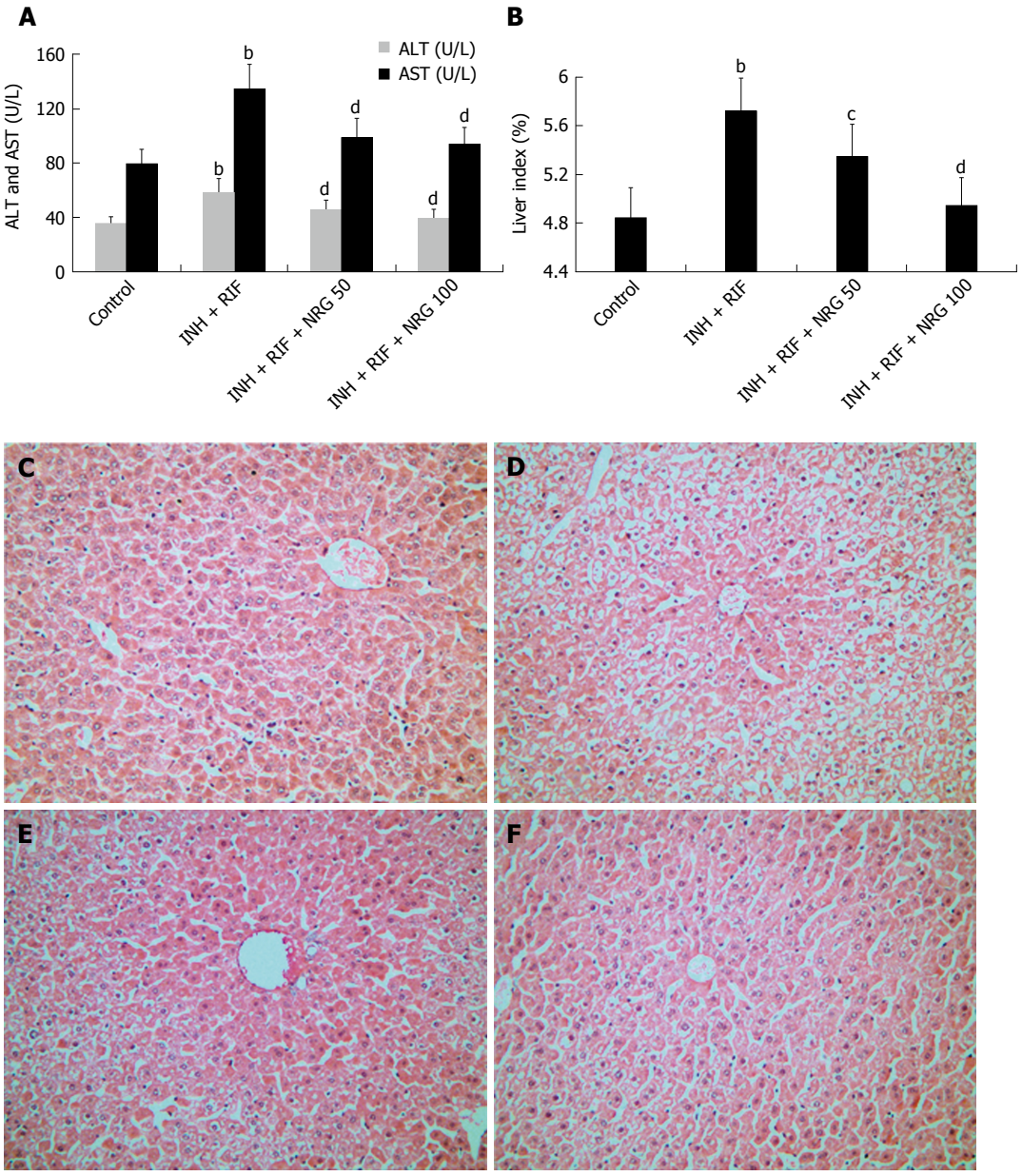

Figure 1 Naringenin pretreatment protects mice from isoniazid- and rifampicin-induced liver injury. A: Levels of serum transaminases were analyzed; B: Liver indexes were measured. All data are expressed as means ± SEM for ten samples per group. $^{b}P < 0.01$ *vs* control group, $^{c}P < 0.05$ *vs* INH and RIF group, $^{d}P < 0.01$ *vs* INH and RIF group; C: Liver section from mice treated with NSS; D: Liver section from mice treated with INH and RIF; E and F: Liver sections from mice pretreated with NRG (50 or 100 mg/kg) before INH and RIF challenge. Liver sections were stained with hematoxylin and eosin (original magnification: × 400). NRG: Naringenin; INH: Isoniazid; RIF: Rifampicin.

RIF-induced elevation of the liver index in a dose-dependent manner (5.34% ± 0.26%, 4.93% ± 0.25% *vs* 5.71% ± 0.28%, $P < 0.01$ or 0.05). Furthermore, HE staining was performed to observe the protective effects of NRG against INH and RIF. As shown in Figure 1C-F, histopathological evaluation showed obvious necrosis and inflammatory cell infiltration in liver of mice administered with INH and RIF. Conversely, mice pretreated with NRG showed minor hepatic injury.

NRG pretreatment reduces INH- and RIF-induced oxidative stress

To investigate the protective effects of NRG against INH- and RIF-induced oxidative stress, levels of GSH, SOD and MDA in the liver tissue were evaluated. As shown in Figure 2A and B, hepatic GSH content and SOD activity were markedly decreased after administration of INH and RIF; however, NRG pretreatment obviously increased INH- and RIF-induced reduction of GSH and SOD (5.91 ± 1.31 mg/gprot, 6.42 ± 1.42 mg/gprot *vs* 3.11 ± 0.73 mg/gprot; 137.31 ± 24.62 U/mgprot, 148.83 ± 26.75 U/mgprot *vs* 102.34 ± 19.22 U/mgprot; all $P < 0.01$). In contrast, as shown in Figure 2C, hepatic MDA content, a marker of lipoperoxide, was significantly increased after administration of INH and RIF, but NRG pretreatment obviously attenuated

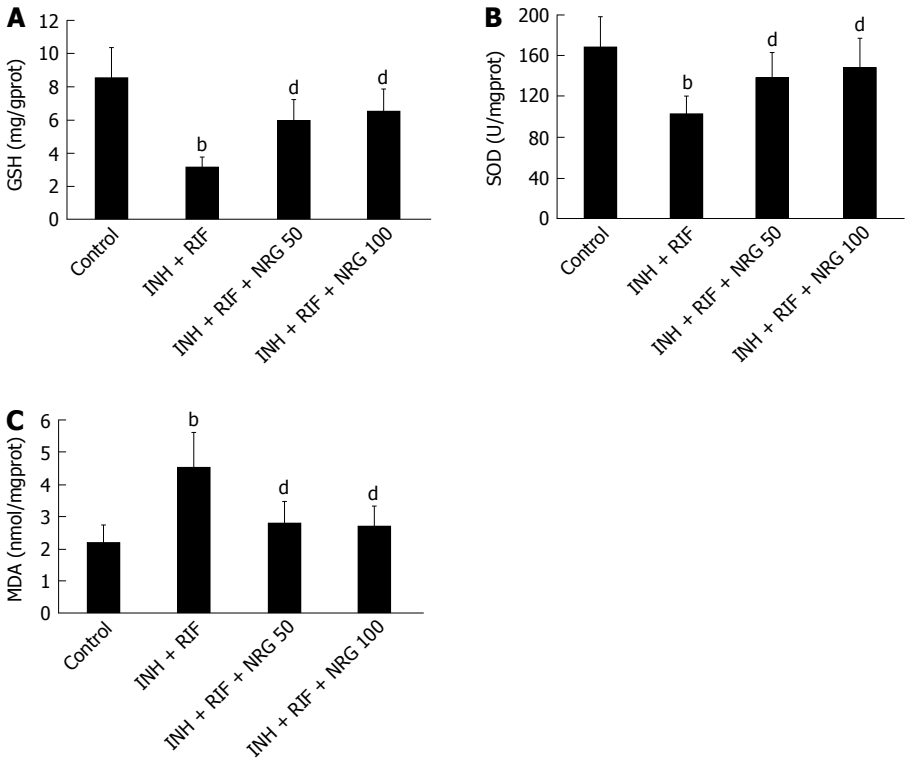

Figure 2 Naringenin pretreatment protects against isoniazid- and rifampicin-induced oxidative stress in liver. A: GSH levels were measured; B: SOD levels were measured; C: MDA levels were measured. All data are expressed as mean ± SEM for ten samples per group. $^{b}P < 0.01$ *vs* control group, $^{d}P < 0.01$ *vs* INH and RIF group. NRG: Naringenin; INH: Isoniazid; RIF: Rifampicin; GSH: Glutathione; SOD: Superoxide dismutase; MDA: Malondialdehyde.

INH- and RIF-induced elevation of MDA (2.76 ± 0.67 nmol/mgprot, 2.64 ± 0.64 nmol/mgprot *vs* 4.49 ± 1.12 nmol/mgprot, $P < 0.01$).

NRG pretreatment prevents INH- and RIF-induced apoptosis in hepatocytes

To further investigate the potential mechanisms by which NRG alleviates INH- and RIF-induced liver injury, TUNEL staining was used to estimate the apoptotic changes in hepatocytes. As shown in Figure 3, the number of TUNEL positive cells in liver was obviously increased after INH and RIF administration, while NRG pretreatment dramatically suppressed INH- and RIF-induced hepatocyte apoptosis. In agreement with TUNEL assay, as shown in Figure 4, caspase-3 activity was obviously increased after administration of INH and RIF, and NRG pretreatment markedly prevented caspase-3 activity induced by INH and RIF. In addition, expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 were measured to investigate the effects of NRG against INH and RIF. As shown in Figure 5, the Bax expression level was significantly increased and the Bcl-2 expression level was significantly decreased in mice treated with INH and RIF. Conversely, NRG pretreatment down-regulated Bax expression and up-regulated Bcl-2 expression compared with INH- and RIF-treated mice (1.25 ± 0.09, 0.99 ± 0.07 *vs* 2.16 ± 0.15; 1.23 ± 0.08, 1.35 ± 0.08 *vs* 0.85 ± 0.06; all $P < 0.01$).

DISCUSSION

Anti-TB drugs are considered as potentially hepatotoxic and can induce hepatic injury. The co-administration of INH and RIF generates metabolic and morphological changes in hepatic tissue because of the fact that the liver is the primary detoxifying organ for the anti-TB drugs[2]. In this study, the liver injury mouse model was developed successfully *via* intragastric infusion of INH and RIF for 14 d; results show that serum transaminases levels of INH- and RIF-treated mice are significantly increased as compared to those in normal control mice.

NRG is considered to have beneficial effects on human health and has been vigorously researched as a potential candidate to protect against various liver diseases[18,19]. Previous studies have shown that NRG could protect against CCl_4-induced chemical liver injury by reducing the oxidative stress reaction[15]. NRG also was shown to attenuate cholesterol-induced hepatitis in rats through inhibition of the NF-κB signaling pathway[14]. In addition, NRG had been shown to inhibit the hepatitis C virus by silencing its infection pathway[20].

In this study, for the first time, we explored the effects of NRG on INH- and RIF-induced liver injury and the possible underlying mechanisms in mice. We found that NRG pretreatment significantly attenuated INH- and RIF-induced elevation of the liver index, and serum ALT and AST. Furthermore, NRG pretreatment obviously attenuated INH- and RIF-induced pathologic injury.

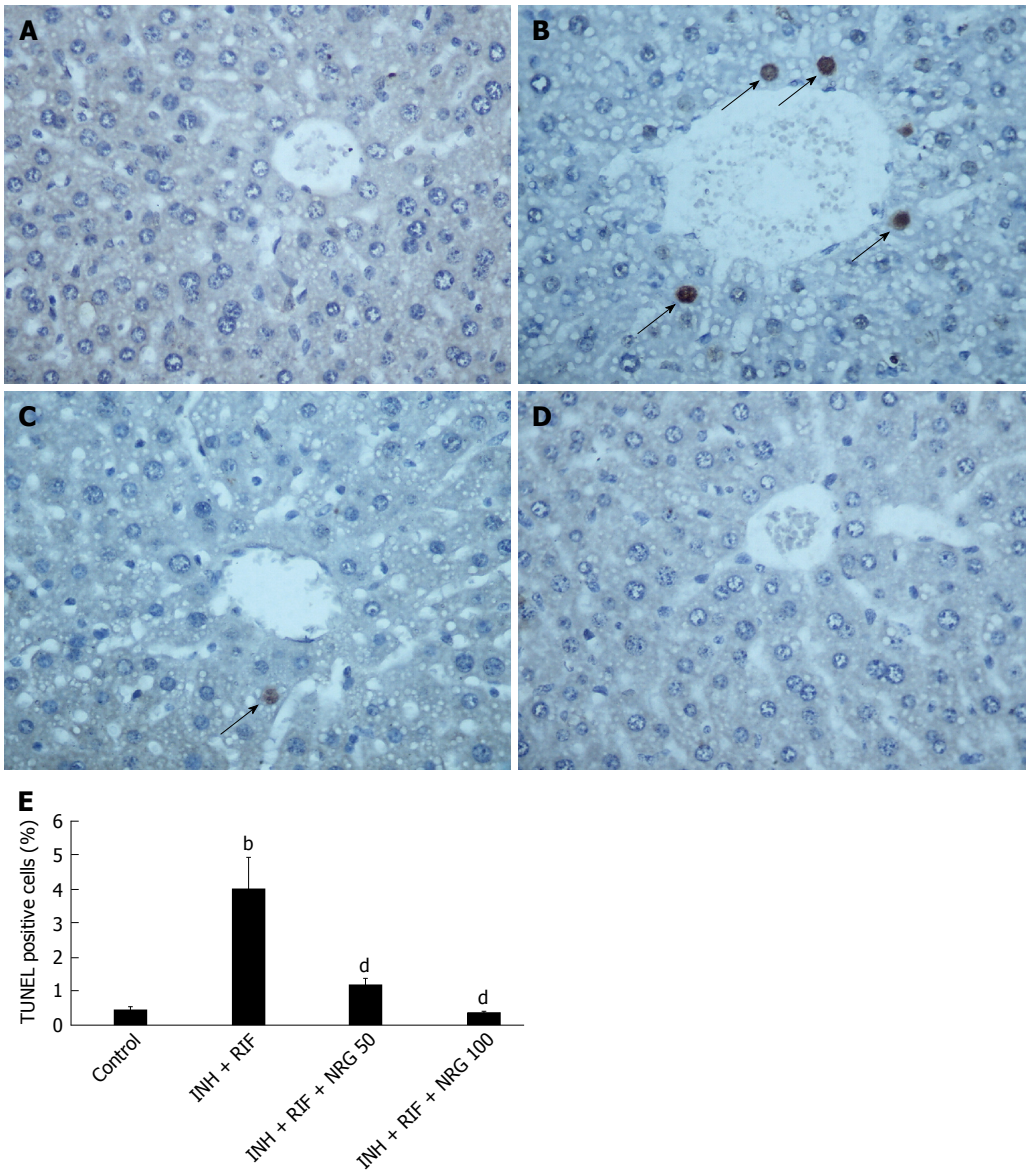

Figure 3 Naringenin pretreatment protects against isoniazid- and rifampicin-induced hepatocytes apoptosis. A-D: Apoptosis was detected with TUNEL staining of liver sections from mice treated with NSS (A), INH and RIF (B), or pretreated with NRG (50 or 100 mg/kg) before INH and RIF challenge (C and D). The number of TUNEL positive cells (arrow) was counted in six randomly selected areas from each tissue section at magnification × 400; E: All data are expressed as mean ± SEM for six samples per group. $^{b}P < 0.01$ *vs* control group, $^{d}P < 0.01$ *vs* INH and RIF group. NRG: Naringenin; INH: Isoniazid; RIF: Rifampicin.

These results indicate hepatoprotective effects of NRG against anti-TB drug-induced hepatic injury.

Earlier studies showed that INH- and RIF-induced liver injury were linked to the occurrence of oxidative stress[21,22]. In this study, we observed that hepatic GSH content and SOD activity were markedly decreased after INH and RIF administration. Conversely, hepatic MDA level, a marker of lipoperoxide, was found to be significantly increased after INH and RIF administration. These results suggest that oxidative stress might play a partial role in INH- and RIF-induced hepatic injury.

NRG has exhibited antioxidant activity against oxidative stress damage. Recent studies showed that NRG could attenuate oxidative stress by decreasing the lipid peroxide level in lead-treated liver and kidney[12]. Additionally, NRG was shown to inhibit lipid peroxidation and thus to protect against the damaging effects of free radicals in acetaminophen-induced acute liver injury[13]. Moreover, NRG was shown to help to increase hepatic GSH and SOD levels and to decrease MDA content, lipid peroxidation and DNA damage in cholesterol-induced hepatic inflammation[14].

In the current study, we observed that NRG pretreatment obviously increased INH- and RIF-induced reduction of GSH and SOD. In contrast, NRG pretreatment obviously attenuated INH- and RIF-induced elevation of MDA. The results of the present study are in accordance with previous reports showing that NRG exerts significant hepatoprotective effects *via* inhibition of oxidative stress.

In this study, we also observed that the percentage of TUNEL positive cells in liver was markedly increased after INH and RIF administration. In agreement with findings from the TUNEL assay, caspase-3 activity was

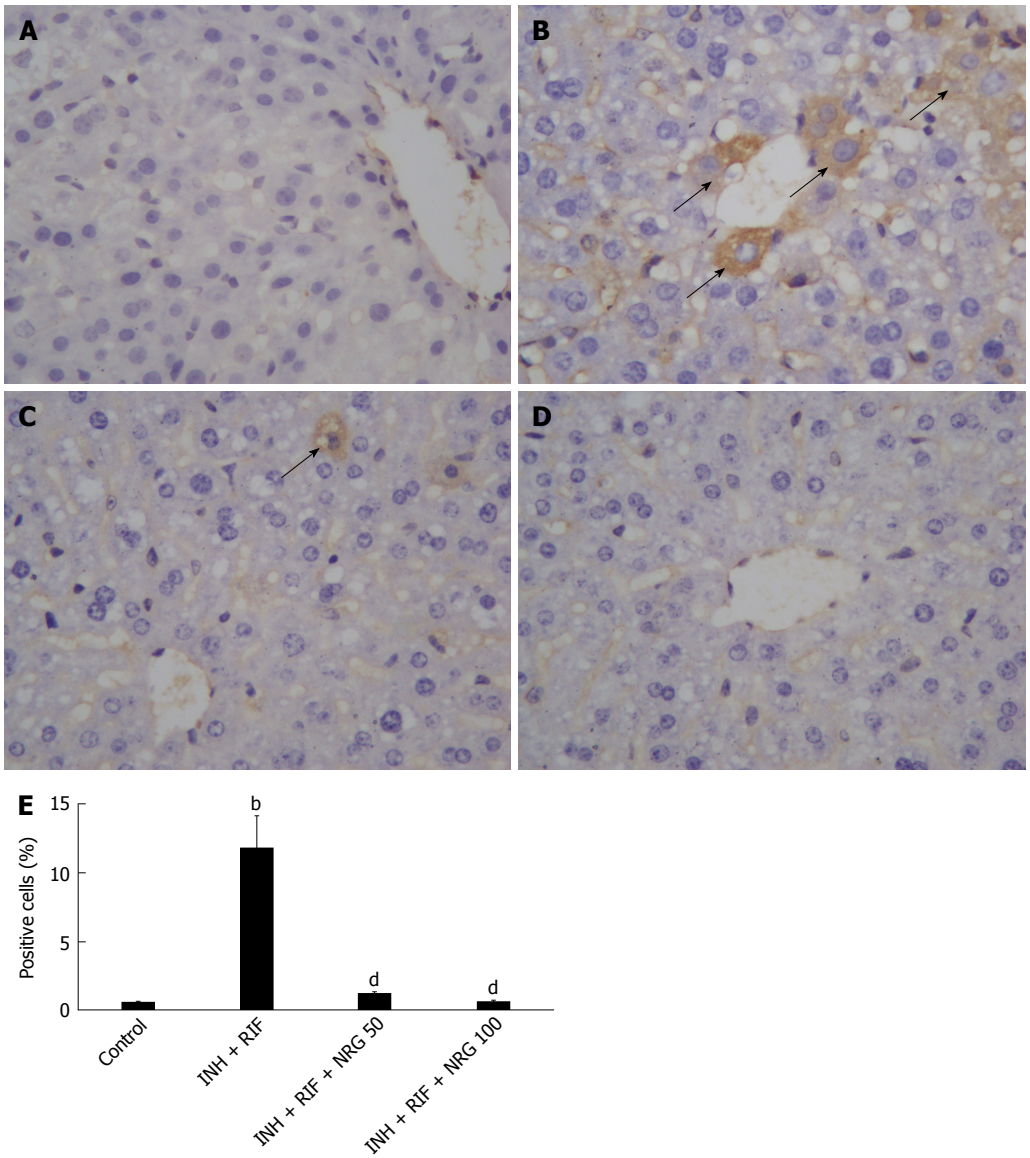

Figure 4 Effect of naringenin on isoniazid- and rifampicin-induced caspase-3 activation. A-D: Caspase-3 activity was measured with immunohistochemical staining of liver sections from mice treated with NSS (A), INH and RIF (B), or pretreated with NRG (50 or 100 mg/kg) before INH and RIF challenge (C and D). The number of cleaved caspase-3-positive cells (arrow) was counted in six randomly selected areas from each tissue section at magnification × 400; E: All data are expressed as mean ± SEM for six samples per group. $^{b}P < 0.01$ *vs* control group, $^{d}P < 0.01$ *vs* INH and RIF group. NRG: Naringenin; INH: Isoniazid; RIF: Rifampicin.

found to be markedly elevated in mice treated with INH and RIF. To further explore the molecular mechanisms of INH- and RIF-induced apoptosis of hepatocytes, the hepatic Bax and Bcl-2 protein expression were examined. Unsurprisingly, the pro-apoptotic protein Bax expression level was significantly increased and anti-apoptotic protein Bcl-2 expression level was significantly decreased after INH and RIF challenge. These data imply that hepatocytes apoptosis might play a partial role in INH- and RIF-induced hepatic injury.

Inhibiting the activation of apoptosis could help prevent liver injury. Earlier studies have suggested that NRG induces apoptosis of human cancer cells, whereas it shows no toxic effect on normal cells at a similar dose[23]. Moreover, recent studies showed that NRG protected human keratinocytes against UVB-induced apoptosis to decrease skin aging and cancer through its anti-apoptotic and anti-lipid peroxidation effects[24]. Additionally, NRG inhibited the activation of the caspase-3 and -9 to prevent hepatocytes from undergoing CCl_4-induced apoptosis[25].

In the present study, we found that NRG pretreatment dramatically suppressed INH- and RIF-induced hepatocyte apoptosis. Moreover, NRG pretreatment markedly prevented caspase-3 activity induced by INH and RIF. These results suggest that NRG could protect against INH- and RIF-induced hepatic injury and the mechanism could be related to the anti-apoptotic effect. However, the underlying mechanisms of NRG-mediated anti-apoptotic effect are not well understood.

A recent study has shown that NRG prevents high glucose-induced mitochondria-mediated apoptosis

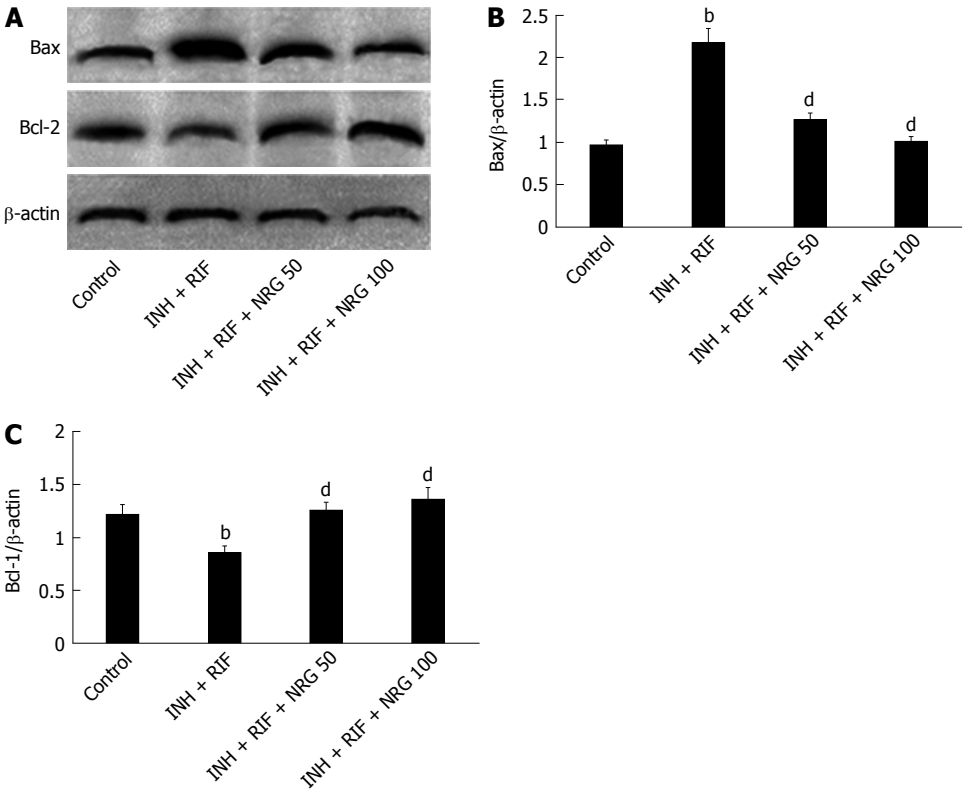

Figure 5 Effect of naringenin on Bax and Bcl-2 expression of isoniazid- and rifampicin-induced liver injury. The protein expression of Bax and Bcl-2 was determined by western blotting. Data are expressed as mean ± SEM for six samples per group. $^{b}P < 0.01$ *vs* control group, $^{d}P < 0.01$ *vs* INH and RIF group. NRG: Naringenin; INH: Isoniazid; RIF: Rifampicin.

involving apoptosis inducing factor, endonuclease-G and caspases. While preventing mitochondrial depolarization, NRG causes a decline in the release of apoptogenic factors like cytochrome C and endonuclease-G from mitochondria, and an increase in expression of anti-apoptogenic factors like Bcl-2[26]. In the present study, we found that NRG pretreatment down-regulated Bax expression and up-regulated Bcl-2 expression, compared with levels found in the INH- and RIF-treated mice. These results indicate that the protective effect of NRG against INH- and RIF-induced hepatocyte apoptosis involves regulation of the Bax and Bcl-2 balance.

In conclusion, the findings of the present study indicate that NRG alleviates INH- and RIF-induced hepatic injury in mice. The protective effect of NRG could be related with suppression of oxidative stress. Moreover, the beneficial effect is associated with its anti-apoptotic effect.

COMMENTS

Background

Isoniazid (INH) and rifampicin (RIF) are the two first-line anti-tuberculosis drugs and are known to be potentially hepatotoxic, with potential for causing drug-induced liver injury. INH is considered to be initiated by cytochrome P450-mediated metabolism of INH to acetylhydrazine and hydrazine, which is hepatotoxic. RIF, which is usually co-administered with INH, is toxic to hepatocytes. Therefore, new and safe preventive measures against INH- and RIF-induced liver injury are urgently needed.

Research frontiers

Previous studies have shown that naringenin (NRG) has wide range of biological and pharmacological activities, such as anti-inflammatory, anti-oxidant and anti-cancer. Related studies have shown that NRG attenuates liver injury induced by lead, acetaminophen, cholesterol overdose and carbon tetrachloride (CCl_4) intoxication. It was suggested that NRG exerts hepatoprotective effects and may have therapeutic value in various liver diseases.

Innovations and breakthroughs

This study is the first to explore the effects of NRG on INH- and RIF-induced liver injury and the possible mechanisms in mice. The results indicate that NRG protects against INH- and RIF-induced oxidative stress and hepatocyte apoptosis.

Terminology

Caspase-3 is a member of the cysteine-aspartic acid protease family. It is activated in the apoptotic cell, both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. It plays an important role in the execution-phase of cell apoptosis.

Applications

NRG widely exists in grapefruit, bitter orange and other plants. It can be processed and purified using modern separation technology and has a broad application prospect.

Peer review

The data show significant protective effect of NRG against anti-tuberculosis drug-induced liver injury through suppression of apoptosis, which is demonstrated by serum transaminases, liver histology, immunohistochemistry

and western blotting. Since liver injury induced by INH and RIF is a serious problem for the treatment of tuberculosis (TB), the data are important and may provide a novel clue for management of TB.

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ORIGINAL ARTICLE

Basic Study

Effects of ω-3 fatty acids on toll-like receptor 4 and nuclear factor-κB p56 in lungs of rats with severe acute pancreatitis

Bin Wang, Xiao-Wei Wu, Mei-Xia Guo, Min-Li Li, Xiao-Bing Xu, Xin-Xin Jin, Xiao-Hua Zhang

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Author contributions: Wang B designed and performed the research and wrote the paper; Wu XW, Xu XB and Jin XX performed the research; Guo MX and Li ML provided analytic tools; Zhang XH designed the paper.

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Institutional animal care and use committee statement: The study was approved by the Institutional Animal Care and Use Committee of Jinling Hospital. All operations were performed according to international guidelines concerning the care and treatment of experimental animals.

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Abstract

AIM

To determine the effects of ω-3 fatty acids (ω-3FA) on the toll-like receptor 4 (TLR4)/nuclear factor κB p56 (NF-κBp56) signal pathway in the lungs of rats with severe acute pancreatitis (SAP).

METHODS

A total of 56 Sprague-Dawley rats were randomly divided into 4 groups: control group, SAP-saline group, SAP-soybean oil group and SAP-ω-3FA group. SAP was induced by the retrograde infusion of sodium taurocholate into the pancreatic duct. The expression of TLR4 and NF-κBp56 in the lungs was evaluated by immunohistochemistry and Western blot analysis. The levels of inflammatory cytokines interleukin-6 and tumor necrosis factor-alpha in the lungs were measured by enzyme-linked immunosorbent assay.

RESULTS

The expression of TLR4 and NF-κBp56 in lungs and of inflammatory cytokines in serum significantly increased in the SAP group compared with the control group ($P < 0.05$), but was significantly decreased in the ω-3FA group compared with the soybean oil group at 12 and 24 h ($P < 0.05$).

CONCLUSION
During the initial stage of SAP, ω-3FA can efficiently lower the inflammatory response and reduce lung injury by triggering the TLR4/NF-κBp56 signal pathway.

Key words: Severe acute pancreatitis; ω-3 fatty acids; Lung injury; Toll-like receptor 4; Nuclear factor-κB p56; Cytokine

Core tip: There is no report about the correlation between ω-3 fatty acids and toll-like receptor 4 (TLR4) expression in lungs of animals with severe acute pancreatitis (SAP). In this study, we investigated the effects of ω-3 fatty acids (ω-3FA) on TLR4 and nuclear factor κB p56 (NF-κBp56) in lungs of rats with SAP and the levels of cytokines in serum to examine the effects of ω-3FA on TLR4 and NF-κBp56 of lungs in rats with SAP.

Wang B, Wu XW, Guo MX, Li ML, Xu XB, Jin XX, Zhang XH. Effects of ω-3 fatty acids on toll-like receptor 4 and nuclear factor-κB p56 in lungs of rats with severe acute pancreatitis. *World J Gastroenterol* 2016; 22(44): 9784-9793 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9784.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9784

INTRODUCTION

Severe acute pancreatitis (SAP) is a critical illness associated with long-term treatment and high mortality. Mortality can approach 50% due to induction of systemic inflammatory response syndrome (SIRS) during the early stages of the disease, subsequently leading to multiple organ dysfunction syndrome (MODS)[1]. Acute lung injury is common, with approximately 20% of patients developing acute respiratory distress syndrome (ARDS). ARDS is a primary cause of death during the early stages of SAP[2,3].

Recent reports have highlighted the role of activation of inflammatory cytokines and signal pathways in pancreatic tissues during the process of SAP[4,5]. Activation of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), and infiltration of inflammatory cells in early SAP can lead to pathological injury not only in pancreatic tissue but also in extra-pancreatic organs, through activation of downstream inflammatory mediators by the amplification of a series of cascade reactions[6]. These injuries can then induce SIRS or even MODS[7,8]. It has been shown that toll-like receptor 4 (TLR4) plays a critical role in the initiation of SAP. TLR4 can regulate the transcription of inflammatory cytokines, which leads to local inflammation in multiple systems and/or organs[9-12].

Toll-like receptors (TLRs) are key modulators of the innate immune response. Members of the TLR family recognize and bind to their corresponding ligand to activate signal transduction pathways and thus produce different biological functions in response to various stimuli[13]. TLR4 is the first reported TLR by which the mediated signal pathway can non-specifically bind in pathogen-associated molecular patterns[14]. Nuclear factor-κB p56 (NF-κBp56), a nuclear transcription factor present in a wide variety of cells, shares an upstream/downstream relationship with TLR4[15]. NF-κBp56 mainly functions at the level of regulating inflammation, cell survival, and apoptosis[16].

Under normal circumstances, members of NF-κBp56 form homomeric or heteromeric dimers in cytoplasm, which exert their function on the activation/inhibition of transcription[17]. Following activation of the TLR4-mediated signal transduction pathway, NF-κBp56 activation and transcription of related inflammatory cytokines are then stimulated[18]. Several studies have confirmed that the expression and activation of TLR4 and NF-κBp56 were up-regulated, and a large number of inflammatory cytokines were detected in the SAP rat model induced in a variety of ways[19-21].

Thus, it is highly likely that the TLR4/NF-κBp56 signal pathway is closely related to the occurrence and development of SAP. The global inflammation in SAP is an important step in the initiation of MODS, in which TLR4 functions as a "messenger"[22]. During the onset stage of SAP, through TLR4, local inflammation mediates the activation of various inflammatory cytokines, which in turn spread to other organs and lead to inflammation of multiple organs[23].

Parenteral nutrition support with ω-3 fatty acids (ω-3FA) alters cytokine production and reduces the rate of complications, for example, the duration of mechanical ventilation and the prevalence of nosocomial infections[24-26]. Thus, ω-3FA, which are major components of fish oil-supplemented parenteral nutrition, offers a potential positive effect on the anti-inflammatory response. Recent animal studies have also shown that ω-3FA can have an anti-inflammatory role in pancreatitis[27-29]. However, there are no data on the correlation between ω-3FA and the TLR4/NF-κBp56 signal pathway in the lungs of subjects with SAP. In this study, we investigated the effects of ω-3FA on the expression of TLR4 and NF-κBp56 in the lungs of rats with SAP, as well as the levels of cytokines in lungs.

MATERIALS AND METHODS

Experimental animal model and grouping

All experiments were approved by the Ethics Committee for Animal Research at Jinling Hospital, Nanjing, China. Healthy adult male Sprague-Dawley rats, weighing 230-250 g, were provided by the Experimental Animal Center of Jinling Hospital. All 56 animals were randomly divided into 4 groups: healthy controls (n = 8); SAP-

saline group (n = 16); SAP-soybean oil group (n = 16); and SAP-ω-3FA group (n = 16). Under pentobarbital anesthesia (50 mg/kg body weight), a laparotomy was performed and 5% sodium taurocholate in distilled water (1 mL/kg body weight) was injected into the bilio-pancreatic duct at the rate of 0.2 mL/min using a micro-infusion pump. Controls received an intraductal infusion of saline (0.2 mL/min)[30]. After SAP induction, the SAP-ω-3FA group received an intravenous injection of a combination of the soybean-based compound and 0.2 g/kg fish oil (Omegaven; Fresenius, Bad Homburg, Germany). The SAP-soybean oil group received a soybean-based fat solution without additional fish oil [Intralipid medium-chain triglyceride/long-chain triglyceride 20%; Braun, Melsungen, Germany], and the SAP-saline group received the same volume of saline.

Eight animals from each group were sequentially killed after 12 and 24 h (SAP-saline group, SAP-soybean oil group and SAP-ω-3FA group) by a lethal dose of pentobarbital (200 mg/kg intravenous). The entire lung was removed, and a sample was immediately frozen at -80 ℃ for biochemical analysis. The lung was then fixed in 10% formalin in anatomic orientation for histological analysis. The lung tissues and whole blood were obtained for subsequent analysis.

Measurement of amylase levels in serum

Serum measurement of measurement of amylase (AMY) concentration was conducted by an automated HITACHI-7150 analyzer to ensure successful SAP models.

Measurement of TNF-α and IL-6 levels in lungs

TNF-α and IL-6 levels in lungs were measured using a commercial ELISA according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, United States). The levels of TNF-α and IL-6 were found to be a minimum of 7 pg/mL and 60 pg/mL, respectively.

Pathological examination of lungs

The lung tissue was fixed by 40 g/L formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin. All microscopic sections were analyzed blindly. One slide for each rat per group was studied, and 10 random fields per slide were evaluated. Lung histopathological changes were graded on a scale of 0 to 3 (normal 0, mild 1, moderate 2, severe 3) for alveolar and interstitial edema, inflammatory cell infiltration, and alveolar and interstitial hemorrhage[31].

Immunohistochemistry of TLR4 and NF-κBp56 in lung tissues

The S-P method was used to detect the expression of TLR4 and NF-κBp56 proteins in lung tissues. Fixed sections were incubated with the appropriate primary and secondary antibodies. Color reactions were developed using diaminobenzidine solution according to the manufacturer's instructions. Phosphate buffered saline was used as a negative control, in lieu of antibody. For semi-quantitative analyses, areas of positive staining were defined by two independent investigators using Image-Pro 6.0 Plus (MediaCybernetics). Five fields of view for each section were randomly selected, images acquired, and integrated optical density (IOD) determined [density (mean) = IOD/area].

Western blot of TLR4 and NF-κBp56 in lung tissues

Lung tissue samples were homogenized with lysate buffer (10 mmol/L Tris at pH 7.5, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5% Triton-X 100, 0.02 mmol/L NaN3, and 0.2 mmol/L phenylmethanesulphonylfluoride), treated with a sodium dodecyl sulfate-polyacrylamide loading buffer at 95 ℃ for 5 min, and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated protein was transblotted from the gel to the polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA, United States) at 300 mA for 1.5 h at 4 ℃. The membrane was blocked with 5% non-fat dried milk in Tris-buffered saline with 0.05% Tween20 (TBST) for 1 h at room temperature, washed 3 times for 10 min each time in TBST, and incubated with a primary antibody in a 1:500 dilution of goat anti-rat TLR4 and anti-rat NF-κBp56 monoclonal antibody (Upstate, Charlottesville, VA, United States) in TBST containing 5% non-fat dried milk for 2 h at room temperature. After washing 3 times with TBST for 10 min each time, the membranes were incubated with a 1:5000 dilution of peroxidase-conjugated goat anti-rat immunoglobulin G (Sigma-Aldrich) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced fluorescence system (Pierce Biotechnology, Rockford, IL, United States).

Statistical analysis

The results for AMY, cytokine and histopathology score of the lungs represent the mean of 8 experiments and are expressed as mean ± SE. Experimental data from different groups were compared using the non-paired Student's t-test and one-way analysis of variance. The western blot data were from 8 experiments and are expressed as mean optical density, which was analyzed using the Mann-Whitney nonparametric U test. A P value less than 0.05 (2-tailed) was considered statistically significant. All tests were performed using the statistical package SPSS software 11.0 (SPSS, Chicago, IL, United States) in Windows 7.

RESULTS

Serum levels of AMY in rats

Serum AMY was determined and showed a consistent change. Hyperamylasemia was found in all SAP groups, and AMY levels were significantly higher than those in the control group (Table 1).

Table 1 Serum amylase levels in the different groups

Group	Time	Cases	Mean ± SE (IU/L)
Control		8	1316.34 ± 115.21
SAP-saline	12 h	8	4637.11 ± 621.57[a]
	24 h	8	4352.54 ± 436.41[d]
SAP-soybean oil	12 h	8	4978.25 ± 918.48[b]
	24 h	8	4245.89 ± 646.27[e]
SAP-ω-3FA	12 h	8	4638.63 ± 586.14[c]
	24 h	8	4163.47 ± 653.64[f]

[a]$P < 0.05$; [d]$P < 0.05$; [b]$P < 0.01$; [e]$P < 0.05$; [c]$P < 0.05$; [f]$P < 0.05$ *vs* control group. SAP: Severe acute pancreatitis; ω-3FA: ω-3 fatty acids.

Measurement of TNF-α and IL-6 levels in lungs

Compared with the control group, TNF-α and IL-6 levels in the lungs of the SAP-saline group, SAP-soybean oil group and SAP-ω-3FA group were significantly higher at each time point ($P < 0.05$). Moreover, lung TNF-α and IL-6 levels in the SAP-ω-3FA group were lower than those in the SAP-soybean oil group at each time point ($P < 0.05$) (Table 2).

Histopathology of lung tissues

Significantly greater pulmonary interstitial alveolar edema, and a larger number of alveolar bleeding inflammatory cells were detected in the lungs of the SAP-saline, SAP-soybean oil and SAP-ω-3FA groups, but the progression of inflammation was different among the groups: lung inflammation was significantly decreased in the SAP-ω-3FA group compared with the SAP-soybean oil group at the same time point ($P < 0.05$); no significant differences in histopathological changes were detected between the SAP-saline group and the SAP-soybean oil group at each time point; and no histopathological changes were detected in the control group (Figure 1, Table 3).

Immunohistochemistry of TLR4 and NF-κBp56 in lung tissues

The immunohistochemistry results indicated that TLR4 and NF-κBp56 expression in the lungs was mainly in neutrophils, macrophages, bronchial epithelial cells, alveolar epithelial cells and microvascular endothelium cells. The TLR4 and NF-κBp56 positive rate was significantly higher in the SAP-soybean oil group than in the SAP-ω-3FA group ($P < 0.05$) (Figures 2 and 3).

Western blot of TLR4 and NF-κBp56 in lung tissues

The Western blotting results showed the expression level of TLR4 and NF-κBp56 in the lungs of the SAP-saline, SAP-soybean oil and SAP-ω-3FA groups compared with the control group at 12 and 24 h. The expression levels of both proteins in the groups are shown in Figure 4 and Table 4. Compared with the control group, the SAP-saline group and SAP-soybean oil group showed a significantly higher expression level of TLR4 in the lungs at each time point ($P < 0.05$). TLR4 expression was much lower in the SAP-ω-3FA group. Furthermore, NF-κBp56 expression level was also significantly increased in the SAP groups compared with the control group, while NF-κBp56 expression in the SAP-ω-3FA group was a little lower than that in the SAP-soybean oil group ($P < 0.05$). These results suggest that while the expression of TLR4 and NF-κBp56 significantly increased in the lungs of rats with SAP, ω-3FA down-regulated TLR4 expression and inhibited NF-κBp56 expression in the lungs of rats with SAP.

DISCUSSION

The first sign of multiple organ failure in SAP is often impaired lung function due to ARDS[32]. As a consequence of overactive SIRS, TNF-α and IL-6 are activated within the circulation and attack the pulmonary vascular endothelium[33,34]. Inflammatory cytokines play an important role in lung injury in SAP and in the translation of local pancreatic damage to a systemic inflammatory response[35,36]. The increase in lung pathological damage and lung edema is evidence of SAP-associated lung injury. TLRs are proteins that can trigger the inflammatory cascade reaction. Therefore, an investigation into the tissue-specific expression of TLRs (mainly TLR4) in lungs and determining their roles is of great significance in understanding the pathogenesis of SAP.

It is currently thought that TLRs may play a central role in the recognition of endogenous or exogenous antigens in the immune system and in the initiation of signal transduction in the inflammatory reaction during SAP. Some researchers believe that TLR4 may play an important role in the synthesis and release of inflammatory cytokines, and up-regulation of the TLR4 gene may be related to the development and progression of organ injury during SAP[37,38]. Some studies have indicated that when SAP is stimulated, the expression of cytokines and cell adhesion molecules is significantly up-regulated in the pancreas, thereby promoting the accumulation of excessive neutrophils in the area of inflammation leading to injury of the pancreas and other organs[39,40]. Our study showed that, during the early stages of SAP, there was obvious expression of TLR4 and NF-κBp56 in lung tissues. This expression increased as SAP developed; peak expression was seen at 24 h. Changes in the concentration of TNF-α and IL-6 in lungs were consistent with pathological changes in lung tissues.

In recent years, a new ω-3 fish oil emulsion was found to not only supply energy but also to exert some important biological effects, such as immune regulation and organ protection[41-43]. The main mechanisms of action are as follows. On the one hand, ω-3FA can enter the phospholipid pool of the cell membrane to replace ω-6FA, and reach an appropriate ω-3/ω-6 ratio

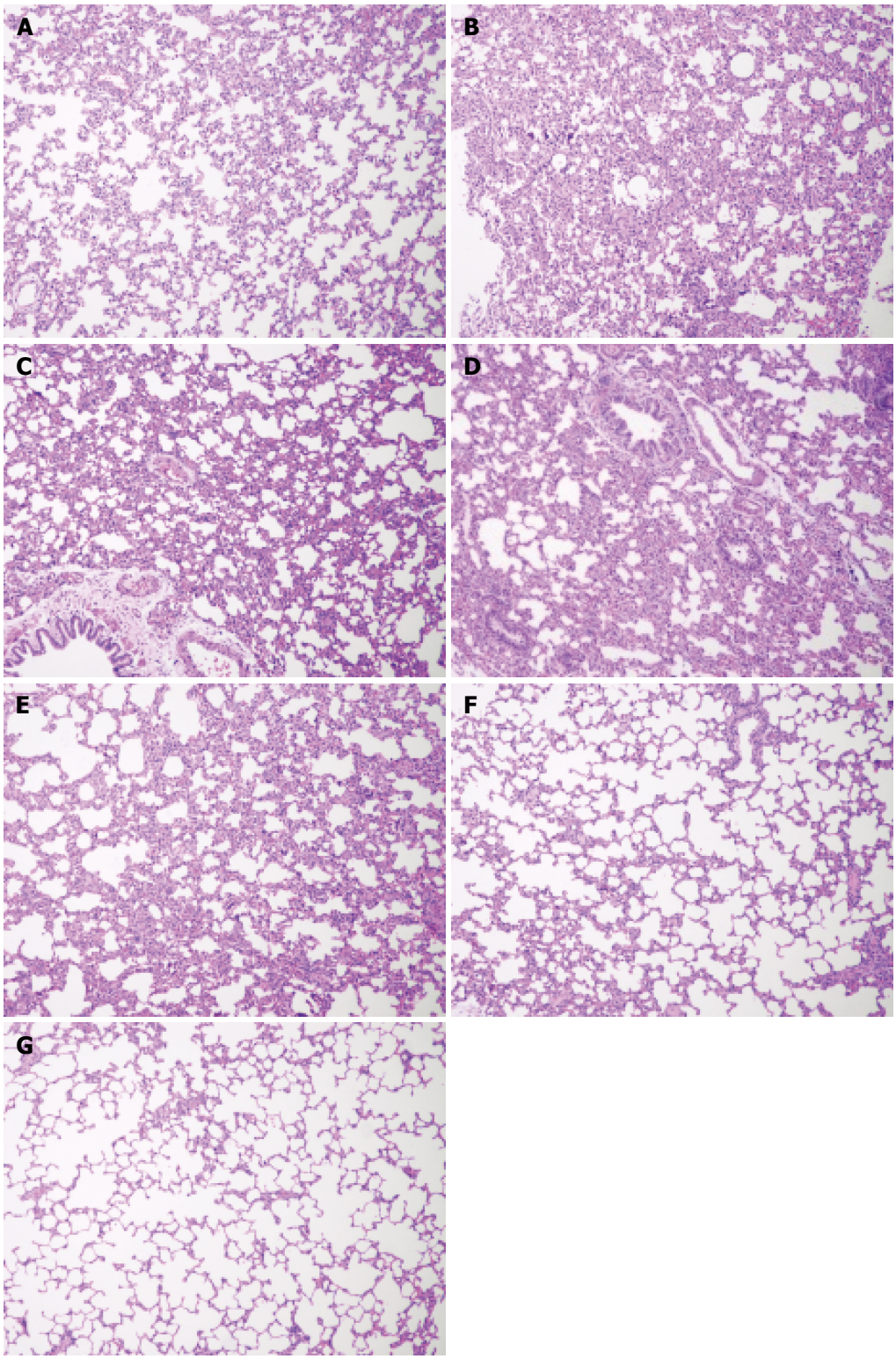

Figure 1 Representative photographs of lung histology in rats from each group (magnification × 200). A: HC group; B: SAP-saline 12 h group; C: SAP-saline 24 h group; D: SAP-soybean oil 12 h group; E: SAP-soybean oil 24 h group; F: SAP-ω-3FA 12 h group; G: SAP-ω-3FA 24 h group. HC: Healthy control; SAP: Severe acute pancreatitis; ω-3FA: ω-3 fatty acids.

(1:2 to 1:4). They can competitively combine with cyclooxygenase and lipoxygenase to inhibit production of inflammatory mediators from ω-6FA which have a strong pro-inflammatory action and immune regulation. On the other hand, by reducing the expression of surface receptors and molecules in immune cell membranes, ω-3FA can participate in the immune response, reduce the activity of immune cells and the production of cytokines, and inhibit the inflammatory response[44-46]. In our study, ω-3FA reduced the release

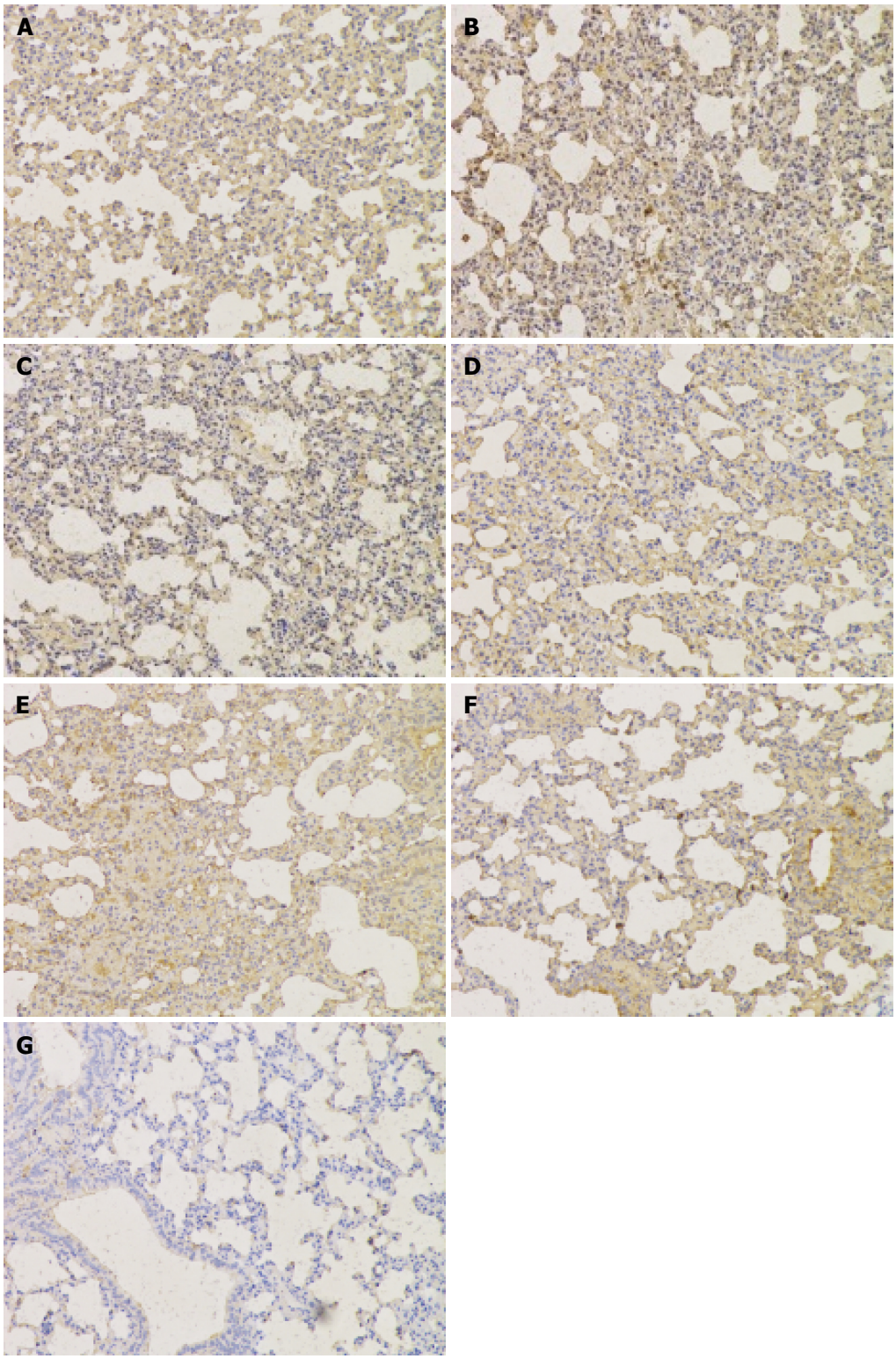

Figure 2 Immunohistochemical analysis of the toll-like receptor 4 protein in lungs from each group (magnification × 200). A: HC group; B: SAP-saline 12 h group; C: SAP-saline 24 h group; D: SAP-soybean oil 12 h group; E: SAP-soybean oil 24 h group; F: SAP-ω-3FA 12 h group; G: SAP-ω-3FA 24 h group. HC: Healthy control; SAP: Severe acute pancreatitis; ω-3FA: ω-3 fatty acids.

of the inflammatory cytokines TNF-α and IL-6, and reduced lung injury.

However, it is unclear whether ω-3FA inhibits the inflammatory response by reducing the expression of TLR4 in lungs. In our study, we found that the expression of TLR4 and NF-κBp56 was decreased in the lungs of rats treated with ω-3FA. The inflammatory response and pathological changes in the lungs improved after the application of ω-3FA. This suggested that ω-3FA indirectly inhibits the expression of TLR4 by

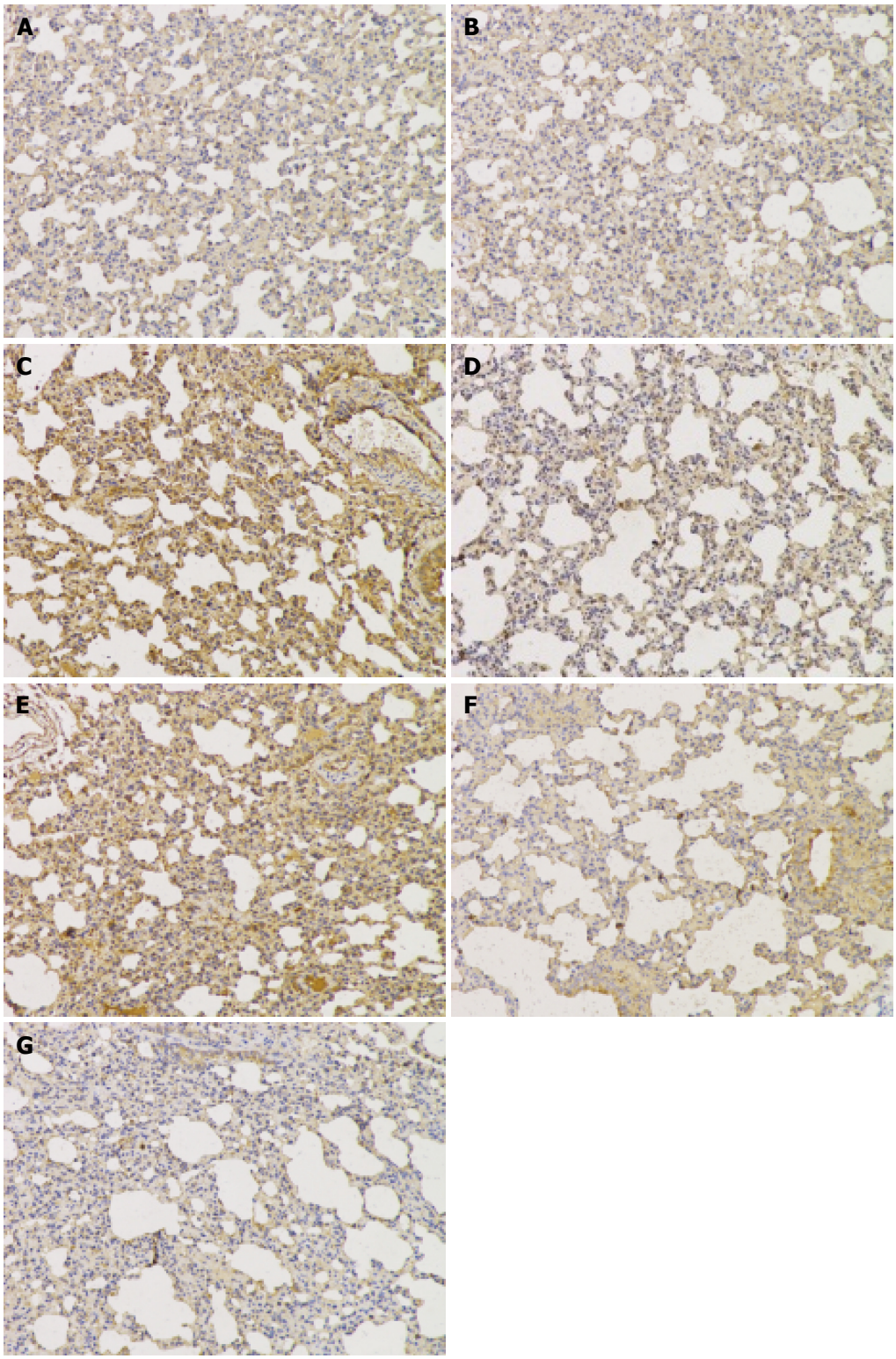

Figure 3 Immunohistochemical analysis of the nuclear factor-κB p56 protein in lungs from each group (magnification × 200). A: HC group; B: SAP-saline 12 h group; C: SAP-saline 24 h group; D: SAP-soybean oil 12 h group; E: SAP-soybean oil 24 h group; F: SAP-ω-3FA 12 h group; G: SAP-ω-3FA 24 h group. HC: Healthy control; SAP: Severe acute pancreatitis; ω-3FA: ω-3 fatty acids.

inhibiting activation of NF-κBp56.

In conclusion, TLR4 may play an important role during SAP-induced lung injury. TLR4 activation results in a variety of inflammatory reactions, and ω-3FA can inhibit the inflammatory response and reduce lung injury by inhibiting the TLR4/NF-κBp56 signal pathway. Development of these therapeutic strategies should help reduce the complications and mortality associated

Table 2 Changes in tumor necrosis factor-alpha and interleukin-6 in the lungs of the groups at each time point (pg/mL, mean ± SE, *n* = 8)

	Control	SAP-saline	SAP-soybean oil	SAP-ω-3FA
TNF-α (12 h)	24.18 ± 1.05	43.24 ± 4.26[a]	46.17 ± 13.63[ad]	33.42 ± 6.37[abc]
IL-6 (12 h)	625.11 ± 124.1	826.31 ± 76.34[a]	827 ± 44.36[ad]	739.67 ± 65.23[abc]
TNF-α (24 h)	24.22 ± 1.03	38.67 ± 4.87[a]	42.45 ± 11.14[ad]	30.37 ± 10.23[abc]
IL-6 (24 h)	623.21 ± 124.3	909.54 ± 26.23[a]	925.18 ± 34.52[ad]	801.27 ± 51.29[abc]

[a]$P < 0.05$ *vs* control group at the same time point; [d]$P > 0.05$, [b]$P < 0.05$ *vs* SAP-saline group; [c]$P < 0.05$ *vs* SAP-soybean oil group. SAP: Severe acute pancreatitis; ω-3FA: ω-3 fatty acids.

Table 3 Lung histopathology score in each group of rats (mean ± SE, *n* = 8)

Group	Pulmonary interstitial edema	Exudation of inflammatory cells	Alveolar bleeding	Alveolar edema
Control	0.32 ± 0.06	0.21 ± 0.01	1.14 ± 0.02	0.14 ± 0.02
SAP-saline				
12 h	1.24 ± 0.11[a]	1.37 ± 0.14[a]	0.87 ± 0.09[a]	1.13 ± 0.08[a]
24 h	2.53 ± 0.21[a]	2.67 ± 0.26[a]	1.12 ± 0.12[a]	2.14 ± 0.11[a]
SAP-soybean oil				
12 h	1.24 ± 0.21[ad]	1.35 ± 0.24[ad]	0.90 ± 0.25[ad]	1.14 ± 0.12[ad]
24 h	2.15 ± 0.28[abc]	2.38 ± 0.30[abc]	0.85 ± 0.15[abc]	1.95 ± 0.21[abc]
SAP-ω-3FA				
12 h	0.94 ± 0.16[abc]	1.12 ± 0.15[abc]	0.72 ± 0.38[abc]	0.94 ± 0.36[abc]
24 h	2.15 ± 0.28[abc]	2.38 ± 0.30[abc]	0.85 ± 0.15[abc]	1.95 ± 0.21[abc]

[a]$P < 0.05$ *vs* the control group; [d]$P > 0.05$, [b]$P < 0.05$ *vs* SAP-saline group at the same time point; [c]$P < 0.05$ *vs* SAP-soybean oil group at the same time point. SAP: Severe acute pancreatitis; ω-3FA: ω-3 fatty acids.

Table 4 Densitometrically quantified toll-like receptor 4 and nuclear factor-κB p56 bands in the lungs of each group of rats, optical density (mean ± SE)

Group	Time	Cases	TLR4	NF-κBp56
Control		8	2.3 ± 0.3	2.5 ± 0.2
SAP-saline	12 h	8	3.9 ± 0.4[a]	4.0 ± 0.3[a]
	24 h	8	4.0 ± 0.2[a]	4.1 ± 0.4[a]
SAP-soybean oil	12 h	8	3.8 ± 0.3[ad]	3.9 ± 0.3[ad]
	24 h	8	3.9 ± 0.4[ad]	3.8 ± 0.2[ad]
SAP-ω-3FA	12 h	8	2.7 ± 0.3[abc]	3.0 ± 0.2[abc]
	24 h	8	3.1 ± 0.4[abc]	2.9 ± 0.4[abc]

[a]$P < 0.05$ *vs* the control group; [d]$P > 0.05$, [b]$P < 0.05$ *vs* SAP-saline group at the same time point; [c]$P < 0.05$ *vs* SAP-soybean oil group at the same time point. NF-κBp56: Nuclear factor-κB p56; SAP: Severe acute pancreatitis; TLR4: Toll-like receptor 4; ω-3FA: ω-3 fatty acids.

with SAP.

COMMENTS

Background

Acute lung injury (ALI) is one of the most common complications of severe acute pancreatitis (SAP) and often occurs in the early stage of the disease, and may progress to adult respiratory distress syndrome. Toll-like receptor 4 (TLR4) can regulate the transcription of inflammatory cytokines, which leads to ALI.

Research frontiers

It has been found that ω-3 fatty acids (ω-3FA) reduces the release of the inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), and reduces lung injury. It is unclear whether ω-3FA can inhibit the inflammatory response by reducing expression of TLR4 in the lungs.

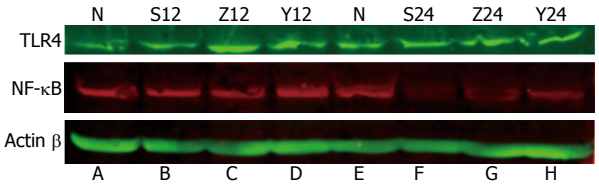

Figure 4 Western blot analysis of the toll-like receptor 4 and nuclear factor-κB protein levels in lungs from each group. A: HC group; B: SAP-saline 12 h group; C: SAP-soybean oil 12 h group; D: SAP-ω-3FA 12 h group; E: HC group; F: SAP-saline 24 h group; G: SAP-soybean oil 24 h group; H: SAP-ω-3FA 24 h group. HC: Healthy control; SAP: Severe acute pancreatitis; ω-3FA: ω-3 fatty acids.

Innovations and breakthroughs

In this study, the authors found that ω-3FA inhibited the inflammatory response and reduced lung injury by inhibiting the TLR4/nuclear factor-κB p56 (NF-κBp56) signal pathway.

Applications

The findings of this study may help to further understand the role of TLR4 during the course of SAP-induced lung injury.

Peer-review

This is a well-intentioned basic study that has evaluated the effects of ω-3FA on expression of TLR4 and NF-κBp56 of lungs with SAP and the levels of cytokines in rat.

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ORIGINAL ARTICLE

Retrospective Study

Association between *Helicobacter pylori* status and metachronous gastric cancer after endoscopic resection

Sung Bum Kim, Si Hyung Lee, Seung Il Bae, Yo Han Jeong, Se Hoon Sohn, Kyeong Ok Kim, Byung Ik Jang, Tae Nyeun Kim

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Author contributions: Lee SH, Kim KO, Jang BI and Kim TN designed research and supervised the report; Jeong YH, Sohn SH, Bae SI and Kim SB analyzed the data; Kim SB and Bae SI wrote the paper.

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: We have no conflict-of-interest to disclose.

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Abstract

AIM

To investigate the effect of *Helicobacter pylori* (*H. pylori*) status test and *H. pylori* eradication on the occurrence of metachronous gastric cancer (MGC) after endoscopic submucosal dissection (ESD) of early gastric cancer (EGC) and risk factors of MGC.

METHODS

The authors retrospectively reviewed the medical records of 433 patients (441 lesions) who underwent ESD for EGC from January 2005 to January 2015 in Yeungnam University Hospital. Patients were categorized into two groups; the *H. pylori* tested group (n = 257) and the *H. pylori* non-tested group (n = 176) based on performance of *H. pylori* status test after ESD of EGC. The *H. pylori* tested group was further categorized into three subgroups based on *H. pylori* status; the *H. pylori*-eradicated subgroup (n = 120), the *H. pylori*-persistent subgroup (n = 42), and the *H. pylori*-negative subgroup (n = 95). Incidences of MGC and risk factors of MGC were identified.

RESULTS

Median follow-up duration after ESD was 30.00 mo (range, 6-107 mo). Total 15 patients developed MGC during follow-up. MGC developed in 11 patients of the *H. pylori* tested group (7 in the *H. pylori*-negative subgroup, 3 in the *H. pylori*-eradicated subgroup, and 1 in the *H. pylori*-persistent subgroup) and 4 patients

of the *H. pylori* non-tested group (P > 0.05). The risk factors of MGC were endoscopic mucosal atrophy in the *H. pylori* tested group and intestinal metaplasia in all patients.

CONCLUSION

H. pylori eradication and *H. pylori* status test seems to have no preventive effect on the development of MGC after ESD for EGC. The risk factors of MGC development were endoscopic mucosal atrophy in the *H. pylori* tested group alone and intestinal metaplasia in all patients.

Key words: Metachronous gastric cancer; Endoscopic submucosal dissection; *Helicobacter pylori*

Core tip: This is a retrospective study to evaluate the effect of *Helicobacter pylori* (*H. pylori*) status test and *H. pylori* eradication on the occurrence of metachronous gastric cancer (MGC) after endoscopic submucosal dissection (ESD) of early gastric cancer (EGC) and risk factors of MGC. *H. pylori* status test and *H. pylori* eradication seems to have no preventive effect on the occurrence of MGC after ESD for EGC. The risk factors of MGC were endoscopic gastric mucosal atrophy in *H. pylori* tested group alone and intestinal metaplasia in all patients.

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INTRODUCTION

Endoscopic resection (ER) including endoscopic mucosal resection and endoscopic submucosal dissection (ESD) is a recognized as one of treatment options for curative resection of early gastric cancer (EGC) without simultaneous concomitant lymph node metastasis[1-4]. Unlike surgery of EGC, ER preserves most part of the stomach and this leads to increased risk of metachronous gastric cancer (MGC) development in residual gastric mucosa[5]. As more EGCs are treated with ER recently, identifying risk factors of MGC development after ER of EGC is important.

Helicobacter pylori (*H. pylori*) infection is related to the development of gastritis, atrophy, intestinal metaplasia, dysplasia, and gastric cancer[6-9]. Among dietary, environmental, and genetic risk factors of gastric cancer, *H. pylori* is classified as a Group 1 or definite carcinogen for gastric cancer by the World Health Organization[10]. In previous reports, the odds for development of gastric cancer reported to increase by 2-4 folds in the patients with *H. pylori* infection[11,12].

The effect of *H. pylori* eradication on development of MGC after ER of EGC is still on debate. A study of 132 patients who underwent ER for EGC and showed positive *H. pylori* serologic test demonstrated that *H. pylori* eradication inhibited the growth of new gastric cancer[13] and a retrospective study of 283 patients with *H. pylori* infection at time of ESD for EGC showed that failure of *H. pylori* eradication was a risk factor of MGC development[5]. However, a study of 1258 patients who underwent ESD for EGC reported that the incidence rate of MGC was not significantly different between patients with or without *H. pylori* eradication[14] and a retrospective study of 268 patients with a 5-year follow-up reported that *H. pylori* eradication after ER for EGC did not significantly reduced the incidence of MGC[15]. Studies about the effect of *H. pylori* status test on development of MGC after ER of EGC has been scarce.

The aims of this study were to investigate the effect of *H. pylori* status test and *H. pylori* eradication on the occurrence of MGC after ESD of EGC and risk factors of MGC.

MATERIALS AND METHODS

Patients

The medical records of 599 patients with 611 lesions who underwent ESD for EGC from January 2005 to January 2015 at Yeungnam university hospital were retrospectively reviewed. Exclusion criteria of the present study were as follows: additional gastrectomy due to a non-curative ESD of EGC and short-term follow-up duration (< 6 mo) and a total of 166 patients with 170 lesions were excluded from the present study. Finally, 433 patients with 441 lesions were included for analysis. Baseline clinical characteristics of the patients, characteristics and histology findings of EGC, performance of *H. pylori* status test and *H. pylori* eradication and occurrence of MGC were analyzed. Institutional review board approval was obtained for this study (2016-06-035).

H. pylori status and follow-up

Patients were divided into two groups; the *H. pylori* tested group (n = 257) and the *H. pylori* non-tested group (n = 176) based on performance of *H. pylori* status test after ESD of EGC. Patients in the *H. pylori* tested group were further divided into three subgroups; the *H. pylori* negative subgroup, the *H. pylori* eradicated subgroup, and the *H. pylori* persistent subgroup (Figure 1).

Among patients with positive *H. pylori* test results, patients who agreed to treat *H. pylori* infection received *H. pylori* eradication. The regimen for first-line *H. pylori* treatment was triple therapy with amoxicillin 1000 mg, clarithromycin 500 mg, and a proton-pump inhibitor (pantoprazole 40 mg, eomeprazole 40 mg, lansoprazole 30 mg or rabeprazole 20 mg)

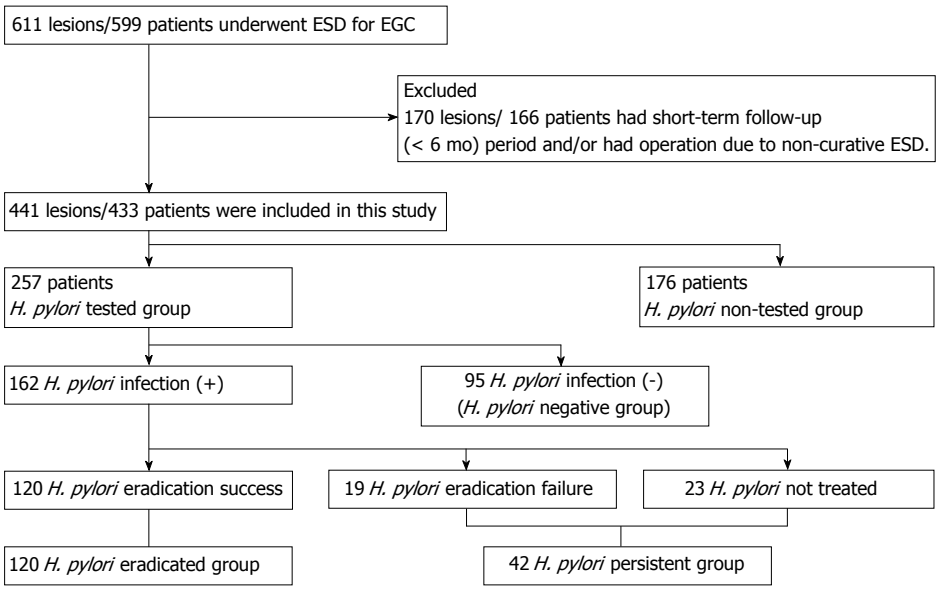

Figure 1 Study flowchart. EGC: Early gastric cancer; ESD: Endoscopic submucosal dissection; *H. pylori*: *Helicobacter pylori*.

all twice daily for a week. The regimen for second-line *H. pylori* treatment was a quadruple therapy with metronidazole 500 mg (3 times daily), tetracycline 500 mg (4 times daily), tripotassium dicitrato bismuthate 300 mg (4 times daily), and a proton-pump inhibitor (twice daily) for 10-14 d. Eradication was confirmed by histology or rapid urase test at scheduled esophagogastroduodenoscopy (EGD) follow-up after ESD or urea breathing test. After ESD, scheduled EGD was performed at 2 or 3, 6, and 12 mo, and annually thereafter.

The presence of gastric mucosal atrophy was assessed through EGD and presence of intestinal metaplasia through histology. MGC was defined as the development of new gastric cancer at a previously uninvolved site in the stomach after the 6 mo following ESD. MGC was confirmed by histology of biopsy specimens. Incidences of MGC was compared according to performance of *H. pylori* status test and among the *H. pylori* eradicated, persistent and negative group and risk factors of MGC were analyzed.

Statistical analysis

Results are presented as means and standard deviations or as medians and ranges. The χ^2 or Fisher's exact test and one-way analysis of variance test or the Student's *t*-test were used to compare categorical and continuous variables, respectively. The log-rank test was used for to compare group incidence rates. Univariate and multivariate Cox proportional hazard regression analyses were used to identify independent risk factors associated with MGC development. Covariates with *P* values of < 0.05 by univariate analyses were entered into multivariate analysis. Statistical analyses of the data were performed using SPSS 20 (IBM SPSS, Chicago, IL, United States). Statistical significance was accepted for *P* values < 0.05.

RESULTS

Baseline characteristics of the patients

Mean age of the 433 patients included in the present study was 67.02 years and 325 (75.1%) patients were male and 108 (24.9%), female. Median follow-up duration after ESD of EGC was 30.00 mo (range, 6-107 mo).

Among 257 patients of the *H. pylori* tested group, 162 (63.0%) patients showed positive result for *H. pylori* test and 95 (37.0%) patients, negative. Of these 162 patients with positive result of *H. pylori* test, *H. pylori* eradication was done in 139 patients and eradication was successful in 120 (86.3%) patients. Ninety-five patients without *H. pylori* infection were classified as the *H. pylori*-negative subgroup, 120 patients with successful *H. pylori* eradication as the *H. pylori*-eradicated subgroup, and 42 patients (19 patients in whom *H. pylori* eradication failed and 23 patients not treated for *H. pylori* infection) as the *H. pylori*-persistent subgroup (Figure 1). The mean age of *H. pylori* tested group was 66.61 years and 189 (73.5%) patients were male. Patients in the *H. pylori*-eradicated subgroup were significantly younger than patients in the *H. pylori*-negative and *H. pylori*-persistent subgroups ($P < 0.05$). The mean follow-up duration was not significantly different between three subgroups ($P > 0.05$). Endoscopic mucosal atrophy and intestinal metaplasia were significantly more prevalent in the *H. pylori*-negative subgroup than the other two subgroups ($P < 0.05$). The location and macroscopic type of primary gastric cancer were not significantly different between three subgroups ($P > 0.05$). The *H. pylori*-persistent subgroup had significantly less differentiated cancers than other two subgroups ($P = 0.032$) (Table 1).

The mean age and follow-up duration were not

Table 1 Baseline characteristics of patients in the *Helicobacter pylori* tested group *n* (%)

	H. pylori negative group (*n* = 95)	*H. pylori* persistent group (*n* = 42)	*H. pylori* eradicated group (*n* = 120)	*P* value
Sex				
Male	74 (77.9)	29 (69.0)	86 (71.7)	0.454
Female	21 (22.1)	13 (31.0)	34 (28.3)	
Age, mean (SD)	68.65 (8.86)	67.31 (9.12)	64.76 (10.10)	0.011
Follow-up period (mo), mean (SD)	36.18 (± 26.74)	33.29 (± 25.93)	32.78 (± 23.72)	0.149
Endoscopic mucosal atrophy	50 (52.6)	8 (19.0)	25 (20.8)	< 0.001
Intestinal metaplasia	63 (66.3)	18 (42.9)	32 (26.7)	< 0.001
Location of primary cancer				
Upper	10 (10.5)	5 (11.9)	10 (8.3)	0.577
Middle	36 (37.9)	14 (33.3)	35 (29.2)	
Lower	49 (51.6)	23 (54.8)	75 (62.5)	
Macroscopic type of primary cancer				
Elevated	40 (42.1)	122 (8.6)	57 (47.5)	0.177
Flat	13 (13.7)	5 (11.9)	9 (7.5)	
Depressed	42 (37.0)	25 (59.5)	54 (45.0)	
Diameter of primary cancer (cm), mean (SD)	14.24 (7.31)	13.83 (6.22)	13.67 (6.90)	0.978
Histology of primary cancer				
Differentiated	93 (97.9)	38 (90.5)	118 (98.3)	0.032
Undifferentiated	2 (2.1)	4 (9.5)	2 (1.7)	
ESD criteria				
Absolute	73 (76.8)	33 (78.6)	95 (79.2)	0.636
Expended	18 (18.9)	5 (11.9)	19 (15.8)	
Beyond expanded	4 (4.2)	4 (9.5)	6 (5.0)	
Depth of primary cancer				
Mucosa	89 (93.7)	40 (95.2)	111 (92.5)	0.819
Submucosa	6 (6.3)	2 (4.8)	9 (7.5)	
Metachronous cancer recurrence	7 (7.4)	1 (2.4)	3 (2.5)	0.173

ESD: Endoscopic submucosal dissection; *H. pylori*: *Helicobacter pylori*.

significantly different between the *H. pylori* tested and the *H. pylori* non-tested groups ($P > 0.05$). Endoscopic mucosal atrophy and intestinal metaplasia was significantly more frequent in the *H. pylori* tested group than in the *H. pylori* non-tested group, and location of primary gastric cancer location was significantly lower in the *H. pylori* tested group than the *H. pylori* non-tested group ($P < 0.05$). In addition, the *H. pylori* non-tested group had more elevated lesions than the *H. pylori* tested group and the *H. pylori* tested group had more depressed lesions than the *H. pylori* non-tested group ($P < 0.05$) (Table 2).

Development of MGC according to H. pylori status

Among total 433 patients, MGC developed in 15 (3.5%) patients; 11 (4.3%) patients in the *H. pylori* tested group and 4 (2.3%) in the *H. pylori* non-tested group without significant difference ($P = 0.262$) (Table 2).

Among 11 patients who developed MGC in the *H. pylori* tested group, MGC developed in 7 (7.4%) patients of the *H. pylori*-negative subgroup, 3 (2.5%) patients of the *H. pylori*-eradicated subgroup, and 1 (2.4%) patient of the *H. pylori*-persistent subgroup. Although the incidence of MGC was higher in the *H. pylori*-negative subgroup than other two subgroups, statistical significance was not found among the three subgroups ($P = 0.173$) (Table 1).

Characteristics of patients with MGC

Mean age of patients with MGC was 68.93 years and all patients with MGC were male. No significant differences were observed between MGC group and non-MGC group in terms of age, primary cancer location, and primary lesion size ($P > 0.05$), and mean follow-up duration was not significantly different between two groups ($P = 0.752$). Endoscopic mucosal atrophy and intestinal metaplasia were significantly more prevalent in patients with MGC than without ($P < 0.05$) (Table 3).

In the *H. pylori* tested group, age, primary cancer location, and lesion size were not significantly different between patients with or without MGC and follow-up duration was similar between two groups (33.72 ± 23.64 *vs* 34.13 ± 25.30, $P = 0.997$). The patient with MGC showed higher proportion of negative *H. pylori* status than without (63.6% *vs* 35.8%, $P = 0.061$). However, endoscopic mucosal atrophy and intestinal metaplasia were observed significantly more in patients with MGC than without (72.7% *vs* 30.5%, $P = 0.003$ and 81.8% *vs* 42.3%, $P = 0.010$) (Table 4).

Factors associated with the development of MGC

In the *H. pylori* test group, endoscopic mucosal atrophy and intestinal metaplasia were found to be significantly associated with the development of MGC by univariate analysis ($P < 0.05$). Multivariate Cox proportional

Table 2 Baseline characteristics of patients according to performance of *Helicobacter pylori* status test *n* (%)

	H. pylori tested group (*n* = 257)	*H. pylori* non-tested group (*n* = 176)	*P* value
Sex			
Male	189 (73.5)	136 (77.3)	0.378
Female	68 (26.5)	40 (22.7)	
Age, mean (SD)	66.61 (9.63)	67.60 (10.03)	0.303
Follow-up period (mo), mean (SD)	34.12 (25.19)	33.31 (18.11)	0.699
Endoscopic mucosal atrophy	83 (32.3)	12 (6.8)	< 0.001
Intestinal metaplasia	113 (44.0)	14 (8.0)	< 0.001
Location of primary cancer			
Upper	25 (9.7)	12 (6.8)	
Middle	85 (33.1)	37 (21.0)	0.006
Lower	147 (57.2)	127 (72.2)	
Macroscopic type of primary cancer			
Elevated	109 (42.4)	95 (54.0)	
Flat	27 (10.5)	24 (13.6)	0.009
Depressed	121 (47.1)	57 (32.4)	
Diameter of primary cancer (cm), mean (SD)	13.91 (6.93)	14.30 (5.74)	0.540
Histology of primary cancer			
Differentiated	249 (96.9)	171 (97.2)	
Undifferentiated	8 (3.1)	5 (2.8)	0.871
ESD criteria			
Absolute	201 (78.2)	142 (80.7)	0.234
Expended	42 (16.3)	20 (11.4)	
Beyond expanded	14 (5.4)	14 (8.0)	
Depth of primary cancer			
Mucosa	240 (93.4)	161 (91.5)	0.456
Submucosa	17 (6.6)	15 (8.5)	
Metachronous cancer recurrence	11 (4.3)	4 (2.3)	0.262

ESD: Endoscopic submucosal dissection; *H. pylori*: *Helicobacter pylori*.

Table 3 Comparisons of clinical characteristics between patients with or without metachronous gastric cancer in all patients *n* (%)

	Non-metachronous gastric cancer group (*n* = 418)	Metachronous gastric cancer group (*n* = 15)	*P* value
Sex			
Male	310 (74.2)	15 (100)	0.028
Female	108 (25.8)	0 (0)	
Age, mean (SD)	66.95 (9.82)	68.93 (9.35)	0.441
Location of primary cancer			
Upper	35 (8.4)	2 (13.3)	0.163
Middle	115 (27.5)	7 (46.7)	
Lower	268 (64.1)	6 (40.0)	
Lesion size (cm), mean (SD)	14.01 (6.10)	15.60 (13.48)	0.350
Endoscopic mucosal atrophy	87 (20.8)	8 (53.3)	0.003
Intestinal metaplasia	117 (28.0)	10 (66.7)	0.001
Histology of undifferentiated type	12 (2.9)	1 (6.7)	0.397
SM invasion	31 (7.4)	1 (6.7)	0.913
Non-performance of *H. pylori* status test	172 (41.1)	4 (26.7)	0.262
Follow-up period (mo), mean period (SD)	33.72 (22.58)	35.60 (22.68)	0.752

SM: Submucosa; *H. pylori*: *Helicobacter pylori*.

hazard regression analysis revealed an association with MGC development only for endoscopic mucosal atrophy (HR = 6.080, *P* = 0.009) (Table 5).

In all patients, endoscopic mucosal atrophy and intestinal metaplasia were significantly associated with MGC development in univariate analysis and multivariate Cox proportional hazard regression analysis showed an association between intestinal metaplasia and MGC development (HR = 4.67, *P* = 0.006) (Table 6).

DISCUSSION

In the present study, 15 (3.5%) of the 433 patients developed MGC after ESD for EGC and this result was comparable with previous reports[16,17]. MGC occurs more frequently after ER for EGC than surgery (2.5%-14% *vs* 1.8%-5%)[16-19]. This increased risk of MGC after ER for EGC can be partly explained by higher proportion of salvaged stomach in ER than

Table 4 Comparisons of the clinical characteristics of patients with or without matachronous gastric cancer in the *Helicobacter pylori* tested group *n* (%)

	Non-metachronous gastric cancer group (*n* = 246)	Metachronous gastric cancer group (*n* = 11)	*P* value
Sex			
Male	178 (72.4)	11 (100)	0.042
Female	68 (29.6)	0 (0)	
Age, mean (SD)	66.44 (9.66)	70.45 (8.52)	0.177
Location of primary cancer			
Upper	24 (9.8)	1 (9.1)	0.290
Middle	79 (32.1)	6 (54.5)	
Lower	143 (58.1)	4 (36.4)	
Lesion size (cm), mean (SD)	13.75 (6.32)	17.36 (15.45)	0.458
Endoscopic mucosal atrophy	75 (30.5)	8 (72.7)	0.003
Intestinal metaplasia	104 (42.3)	9 (81.8)	0.010
Histology of undifferentiated type	8 (3.3)	0 (0)	0.543
SM invasion	16 (6.5)	1 (9.1)	0.736
Persistent *H. pylori* infection	41 (16.7)	1 (9.1)	0.173
H. pylori negative	88 (35.8)	7 (63.6)	0.061
Follow-up period (mo), mean (SD)	34.13 (25.30)	33.72 (23.64)	0.958

SM: Submucosa; *H. pylori*: *Helicobacter pylori*.

Table 5 Results of univariate and multivariate analysis for factors associated with the development of metachronous gastric cancer in the *Helicobacter pylori* tested group

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age ⩾ 65 yr	1.736	0.449-6.705	0.424			
Endoscopic mucosal atrophy	6.080	1.569-23.556	0.009	6.080	1.569-23.556	0.009
Intestinal metaplasia	6.144	1.300-29.033	0.022	2.654	0.400-17.621	0.312
Histology of undifferentiated type	0.000	0.000	0.999			
SM invasion	1.437	0.173-11.942	0.737			
H. pylori negative	3.142	0.895-11.031	0.074	1.638	0.426-6.299	0.473
H. pylori eradication	0.413	0.107-1.595	0.200			

SM: Submucosa; *H. pylori*: *Helicobacter pylori*.

Table 6 Results of univariate and multivariate analysis for factors associated with the development of metachronous gastric cancer in all patients

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* valve	HR	95%CI	*P* value
Age ⩾ 65 yr	1.317	0.442-3.923	0.620			
Endoscopic mucosal atrophy	4.348	1.535-12.320	0.006	1.966	0.526-7.351	0.315
Intestinal metaplasia	5.145	1.722-15.373	0.003	5.145	1.722-15.373	0.003
Histology of undifferentiated type	2.417	0.293-19.902	0.412			
SM invasion	0.892	0.113-7.007	0.913			
H. pylori non-tested	0.520	0.163-1.660	0.270			

SM: Submucosa; *H. pylori*: *Helicobacter pylori*.

surgery. The mean duration of MGC development from ESD of EGC was 35.6 mo and 3 patients developed MGC after 5 years from initial ESD of EGC. A retrospective study of 1526 patients who underwent ESD for EGC reported that 5-year, 7-year, and 10-year cumulative incidence functions of MGC were 9.5%, 13.1%, and 22.7%, respectively[20]. Meticulous examination at surveillance EGD is needed in patients who underwent ER for EGC and EGD should be done with schedule. Further studies are needed to find optimal schedule for surveillance EGD after ER of EGC.

The chronic inflammation of stomach induced by *H. pylori* infection may lead to mucosal atrophy, intestinal metaplasia, and dysplasia, and risk of developing gastric cancer is increased in patients exhibiting such histologic changes of stomach[21,22]. An animal study has reported that *H. pylori* eradication decreased polyp formation, inflammatory cell infiltration, and cellular proliferation in the gastric mucosa and suggested that *H. pylori* eradication could diminish mucosal alterations

related to gastric carcinogenesis[23]. However, the preventative effect of *H. pylori* eradication on MGC development after ESD for EGC is still on debate. In the present study, the incidence of MGC after ESD of EGC was not significantly different between the *H. pylori*-negative subgroup, the *H. pylori*-eradicated subgroup, and the *H. pylori*-persistent subgroups and *H. pylori* eradication had no preventive effect on the development of MGC after ESD of EGC. However, the *H. pylori*-negative subgroup showed higher tendency towards development of MGC after ESD of EGC than other two subgroups without statistical significance and this might be due to significantly higher proportion of endoscopic mucosal atrophy and intestinal metaplasia in the *H. pylori*-negative subgroup than other two subgroups. The higher proportion of patients with intestinal metaplasia in the *H. pylori*-negative subgroup might have led to false negative result in the *H. pylori* status test.

In the present study, the development of MGC was compared according to performance of *H. pylori* status test and no significant difference in the development of MGC after ESD of EGC was observed between two groups during follow-up. As *H. pylori* status test and *H. pylori* eradication failed to show preventive effect on development of MGC after ESD of EGC in the present study, further large scaled prospective studies are needed to clarify the effect of *H. pylori* status test and *H. pylori* eradication on MGC development in patients who underwent ESD for EGC.

The mucosal atrophy of stomach has been previously reported to contribute to the development of MGC[15]. A study of 100 patients who underwent ESD for EGC reported that the frequency of severe atrophy assessed by histology was higher in the group that developed cancer compared to the group that did not and severity of atrophy was the only independent risk factor of MGC development after *H. pylori* eradication[24]. In the present study, endoscopic mucosal atrophy and intestinal metaplasia were observed more frequently in patients with MGC than in those without. Furthermore, multivariate analysis showed that endoscopic mucosal atrophy in the *H. pylori* tested group and intestinal metaplasia in all patients as a risk factor of MGC development after ESD of EGC.

The effect of *H. pylori* eradication on improvement of mucosal atrophy remains unclear in previous studies[25-28]. A study of 544 patients with EGC reported the preventive effect of *H. pylori* eradication on development of MGC after ER of EGC even in patients with corpus atrophy[29]. However, a large-scale, randomized, and controlled study about 1630 healthy carriers of *H. pylori* infection in China reported that the *H. pylori* carriers with precancerous state defined as presence of mucosal atrophy, intestinal metaplasia, or dysplasia had no preventive effect of *H. pylori* eradication on development of gastric cancer[30]. The ineffectiveness of *H. pylori* eradication on MGC development after ESD of EGC in the present study might have been due to irreversible mucosal atrophic change. Further studies to clarify the effect of *H. pylori* eradication on MGC development according to status of gastric mucosa is needed.

In the present study, all patients who developed MGC were male. A previous study reported that male gender was one of risk factors for MGC development of ESD of EGC[20]. However, male gender was not found as a risk factor of MGC in the present study and further study with longer follow up duration is needed to clarify the effect of gender on development of MGC after ESD of EGC.

The present study has several limitations. First, its retrospective nature study makes selection bias inevitable, as was reflected by differences in the baseline characteristics of patients including atrophy and intestinal metaplasia status. Second, relatively small patients of MGC were included for the analysis, and if more patients with MGC had been included, it is possible that *H. pylori* eradication might have been found to influence MGC development. Third, determination of *H. pylori* infection status was inadequate, and thus, false negative and positive results were possibly included. Forth, we did not examine other causes of mucosal atrophy.

In conclusion, *H. pylori* eradication and *H. pylori* status test seems to have no preventive effect on the development of MGC after ESD for EGC. The risk factors of MGC development after ESD of EGC were gastric mucosal atrophy in *H. pylori* tested group and intestinal metaplasia in all patients.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is related to the development of gastritis, atrophy, intestinal metaplasia, dysplasia, and gastric cancer. The odds for development of gastric cancer reported to increase by 2-4 folds in the patients with *H. pylori* infection in previous studies. The effect of *H. pylori* eradication on development of metachronous gastric cancer (MGC) after endoscopic resection (ER) of early gastric cancer (EGC) is still on debate. Studies about the effect of *H. pylori* status test on development of MGC after ER of EGC has been scarce. In this study, we evaluated the effect of *H. pylori* status test and *H. pylori* eradication on the occurrence of MGC after endoscopic submucosal dissection (ESD) of EGC and risk factors of MGC.

Research frontiers

Studies about the preventive role *of H. pylori* eradication in the development of MGC after ER of EGC showed conflicting results.

Innovations and breakthroughs

In this study, *H. pylori* status test and *H. pylori* eradication seems to have no preventive effect on the development of MGC after ESD for EGC. The risk factors of MGC development were endoscopic gastric mucosal atrophy in *H. pylori* tested group alone and intestinal metaplasia in all patients.

Applications

Due to retrospective nature of the study, further prospective studies to clarify the effect of *H. pylori* status test and *H. pylori* eradication on the occurrence of MGC after ESD of EGC and risk factors of MGC are needed.

Terminology

Early gastric cancer: An adenocarcinoma that is restricted to the mucosa or submucosa of stomach, irrespective of lymph node metastasis.

Peer-review

To provide the comments from peer reviewers that most represent the characteristics, values and significance of the article, and allow the readers to have an objective point of view toward the article.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic implications of *FGFR1* and *MYC* status in esophageal squamous cell carcinoma

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Abstract

AIM

To investigate the clinicopathological features and prognostic implications of combined MYC and fibroblast growth factor receptor 1 (*FGFR1*) status in esophageal squamous cell carcinomas (ESCCs).

METHODS

All patients with ESCC (n = 180) underwent surgical resection at Seoul National University Hospital sometime between 2000 and 2013. A tissue microarray was constructed using cores obtained from representative tumor areas of formalin-fixed, paraffin-embedded tissue blocks. *FGFR1* and *MYC* copy numbers were quantified using fluorescence *in situ* hybridization. The level of MYC expression was determined using immunohistochemistry. *FGFR1* and *MYC* amplification status was compared between primary and metastatic lymph nodes. Univariate and multivariate survival analyses were performed according to adjuvant therapy status.

RESULTS

FGFR1 and *MYC* amplifications were observed in 21.4% (37/173) and 54.2% (91/168) of patients, respectively, while MYC expression was observed in 58.9% (106/180) of patients. There was a positive correlation between *MYC* amplification and overexpression (P = 0.002). Although *FGFR1* amplification was not associated with *MYC* amplification or expression, 12.3% (20/163) of patients exhibited both *FGFR1* amplification and MYC expression. There was also a correlation in *FGFR1* amplification status between matched primary tumors and metastatic lymph nodes (P < 0.001). MYC expression was higher in ESCCs with pT1 (P < 0.001) and in those with no lymph node metastasis (P = 0.023). MYC expression was associated with prolonged disease-free survival (P = 0.036) and overall survival (OS) (P = 0.017) but was not an independent prognostic factor. *FGFR1* amplification was an independent predictor for prolonged OS in all patients (P = 0.029) and in those who did not receive adjuvant therapy (P = 0.013). Combined *FGFR1* amplification and MYC expression predicted better OS in patients who did not receive adjuvant therapy (P = 0.034) but not in those who did receive adjuvant therapy.

CONCLUSION

FGFR1 amplification and MYC expression have prognostic implications in resected ESCCs with respect to adjuvant therapy. The role of FGFR1-targeted therapy in ESCC remains to be explored.

Key words: Receptor tyrosine kinase; Fibroblast growth factor receptor 1; MYC; Esophageal squamous cell carcinoma; Gene amplification; Prognosis; Fluorescent *in situ* hybridization

Core tip: MYC expression, together with fibroblast growth factor receptor 1 (*FGFR1)* amplification, was reported to modulate oncogenic transformation. We evaluated both *FGFR1* and MYC statuses in patients with resected esophageal squamous cell carcinoma (ESCC). *FGFR1* and *MYC* amplifications were observed in 21.4% and 54.2% of patients with ESCC, respectively, while 12.3% exhibited both *FGFR1* amplification and MYC expression. MYC expression and *FGFR1* amplification were significantly associated with prolonged survival. Combined *FGFR1* amplification and MYC expression was a predictor of better survival in patients who did not receive adjuvant therapy, but not in those who did. As such, *FGFR1* and MYC might have prognostic implications in resected ESCCs with respect to adjuvant therapy.

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INTRODUCTION

Esophageal cancer is the eighth most common and the sixth leading cause of cancer-related mortality worldwide[1]. Esophageal squamous cell carcinoma (ESCC) accounts for the majority of esophageal cancers. Recently, genomic and molecular alterations have been discovered in ESCC, including activation of the receptor tyrosine kinase (RTK) pathway, cell cycle dysregulation, activation of Wnt and Notch signaling pathways and epigenetic modifications[2,3]. However, molecular targeted therapy for ESCC remains to be established[4].

Fibroblast growth factor receptors (FGFRs) are RTKs expressed in many different cell types and regulate cell proliferation, differentiation and survival. FGFRs also have oncogenic roles in many cancers[5,6]. In contrast, the FGFR signaling pathway can act as a tumor suppressor by promoting cell differentiation, regulating other oncogenic pathways, protecting cells from injury, or mediating immune surveillance[5,7]. *FGFR1* is one of the most frequently amplified genes in ESCC[2,8,9]. Additionally, new drugs targeting *FGFR* and its related pathways, including multi-kinase inhibitors and selected *FGFR* inhibitors, have been introduced for cancer treatment[5,10]. However, the prognostic significance of *FGFR1* amplification in patients with ESCC remains controversial[11,12].

Pulmonary squamous cell carcinoma (SCC) is another cancer frequently showing *FGFR1* amplification. Reportedly, *MYC* expression together with *FGFR1* amplification regulates oncogenic transformation of *FGFR1* and modulates responses to *FGFR* inhibitors in pulmonary SCC[13,14]. Those studies showed that *FGFR1*, located at 8p12, and *MYC*, located at 8q24, were frequently co-amplified in pulmonary SCC[13]. MYC plays an important role in cell proliferation and carcinogenesis in many types of cancer[15,16]. Additionally, a potential role of *MYC* as a predictor of the sensitivity to *FGFR* inhibitors in pulmonary SCC requires further investigation. However, the prevalence of *FGFR1* and *MYC* alterations and their relationship have not been addressed in patients with ESCC. Thus, we investigated *FGFR1* amplification and *MYC* amplification and expression in patients with resected ESCC and analyzed their clinicopathological features and prognostic significance.

MATERIALS AND METHODS

Patients and samples

Patients who underwent surgical resection for ESCC at Seoul National University Hospital (SNUH) from 2000 to 2013 were reviewed. Patients who received neoadjuvant chemo- and/or radiotherapy and those who

Table 1 Clinicopathological characteristics of patients with esophageal squamous cell carcinoma

Variables		*n* (%)
Age (yr)	≤ 60	46 (25.6)
	> 60	134 (74.4)
Sex	Male	169 (93.9)
	Female	11 (6.1)
Smoking	No	28 (15.6)
	Yes	151 (84.4)
Histological grade	WD	35 (19.4)
	MD	119 (66.1)
	PD and basaloid	26 (14.4)
Localization	Upper	7 (3.9)
	Middle	44 (24.9)
	Lower	116 (65.5)
	EGJ	10 (5.6)
T	1a	16 (8.9)
	1b	65 (36.1)
	2	17 (9.4)
	3	78 (43.3)
	4	4 (2.2)
N	0	92 (51.1)
	1	52 (28.9)
	2	29 (16.1)
	3	7 (3.9)
Stage	Ⅰ A	14 (7.8)
	Ⅰ B	49 (27.2)
	Ⅱ A	21 (11.7)
	Ⅱ B	32 (17.8)
	Ⅲ A	37 (20.6)
	Ⅲ B	19 (10.6)
	Ⅲ C	8 (4.4)
Adjuvant therapy	No	112 (67.8)
	Yes	58 (32.2)
FGFR1 amplification	No amplification	136 (78.6)
	Low amplification	3 (1.7)
	High amplification	34 (19.7)
MYC amplification	No amplification	77 (45.9)
	Low amplification	20 (11.9)
	High amplification	71 (42.3)
MYC expression	0 (none)	74 (41.1)
	1 (weak)	54 (30.0)
	2 (moderate)	41 (22.8)
	3 (strong)	11 (6.1)

FGFR1: Fibroblast growth factor receptor 1; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; EGJ: Esophagogastric junction.

had distant metastasis at the time of surgery were excluded. Finally, 180 total patients participated in this study. Clinical data including demographic features, treatment modalities and outcomes were obtained from medical records by an oncologist (B.K.). Overall survival (OS) was calculated from the date of diagnosis to the date of death from any cause or the last follow-up, and disease-free survival (DFS) was calculated from the date of diagnosis to the date of disease recurrence. Pathological tumor-node-metastasis (TNM) stage was based on the 7th American Joint Committee on Cancer. A tissue microarray was constructed from 2-mm diameter cores obtained from representative tumor areas of formalin-fixed paraffin-embedded tissue blocks and submitted for fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC). This study followed the World Medical Association Declaration of Helsinki recommendations and was approved by the Institutional Review Board of SNUH (H-1405-055-579).

FGFR1 and *MYC* FISH

To evaluate *FGFR1* and *MYC* gene copies, FISH was performed using Vysis LSI *FGFR1* SpectrumRed probe, Vysis LSI c-MYC (8q24.12-q24.13) SpectrumOrange probe and Vysis CEP8 (D8Z2) SpectrumGreen probe as a chromosome enumeration probe (Abbott Molecular, Abbott Park, IL, United States), according to the manufacturer's protocol and as reported previously[17]. The entire tumor area was scanned for hot spots representing increased *FGFR1* copy numbers. Random areas were selected for evaluation if the signals were distributed homogeneously. A minimum of 60 non-overlapping tumor nuclei were counted for the number of *FGFR1* and CEP8 signals. *FGFR1* gene copy status was classified according to the criteria proposed by Schildhaus *et al*[18]. In brief, high-level *FGFR1* amplification was defined as follows: (1) an *FGFR1*/CEP8 ratio ≥ 2; (2) ≥ 15 *FGFR1* signals in ≥10% of tumor cells; or (3) average number of *FGFR1* signals/cell ≥ 6. Low-level *FGFR1* amplification was defined as ≥ 5 *FGFR1* signals/cell in ≥ 50% of tumor cells. The same methods and criteria were applied to evaluate *MYC* gene status.

IHC

To evaluate MYC expression, IHC was performed using a rabbit monoclonal anti-*c-MYC* antibody (EP121, Cell Marque, Rocklin, CA, United States) and the Benchmark XT autostainer (Ventana Medical Systems, Tucson, AZ, United States). MYC expression was evaluated using a four-tier scoring system as follows: 0, none or any staining in < 10% of cells; 1, weak; 2, moderate; and 3, strong staining in ≥ 10% of tumor cells. Cases with a score of 1-3 were considered to express MYC.

Statistical analysis

Statistical analysis was performed using SPSS (version 22.0; IBM Corp., Armonk, NY, United States). Differences between *FGFR1* or *MYC* status and clinicopathological variables were determined using Fisher's exact test or Student's *t*-test. The Kaplan-Meier method with the log-rank test and Cox proportional hazards regression analysis were used for survival analyses. Two-sided *P*-values < 0.05 were considered to indicate statistical significance.

RESULTS

Clinicopathological characteristics of patients with ESCC

The clinicopathological features of 180 patients with resected ESCC are summarized in Table 1. Briefly, the

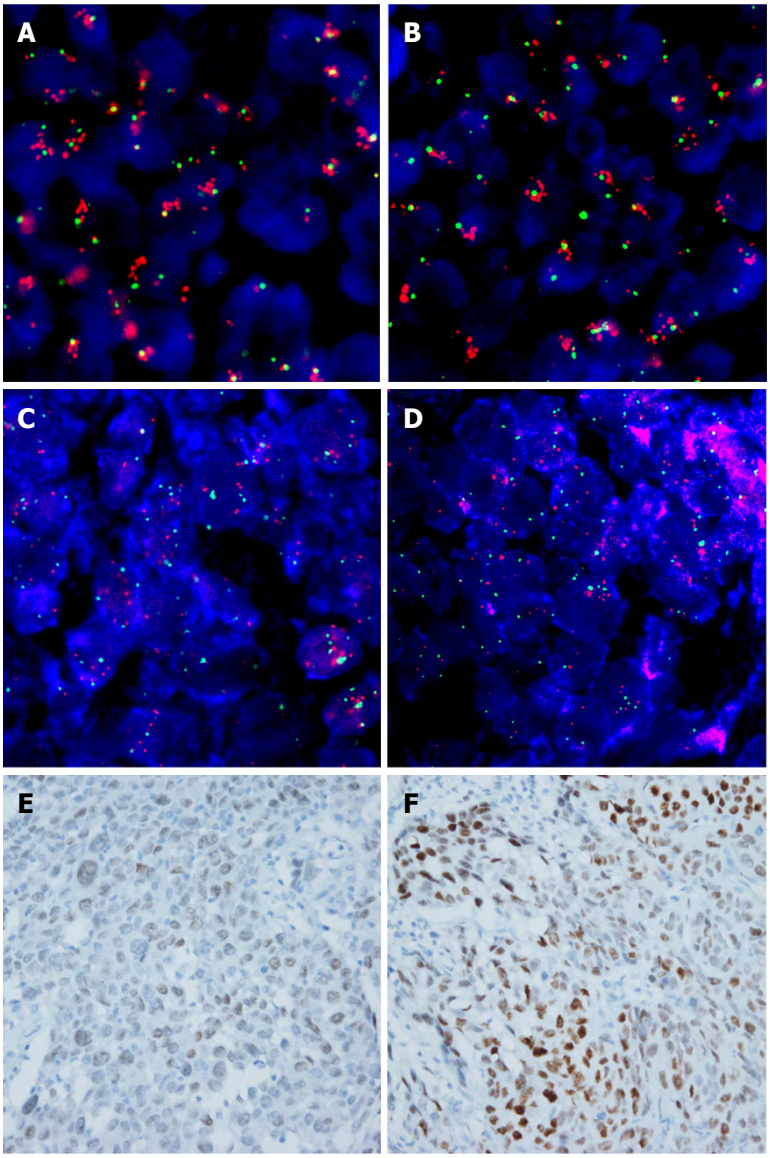


Figure 1 Representative images of fluorescence *in situ* hybridization and immunohistochemistry for fibroblast growth factor receptor 1 and *MYC* in esophageal squamous cell carcinoma. A and B: High amplification of *FGFR1* with increased gene copies of *FGFR1* (red signal), compared to chromosome 8 (CEP8, green signal), was observed; C and D: High amplification of *MYC* with increased gene copies of *MYC* (orange signal), compared to CEP8 was observed; E and F: MYC immunohistochemistry in the nuclei of tumor cells; E: Weak, F: Strong intensity (original magnification, × 400). *FGFR1*: Fibroblast growth factor receptor 1.

median age of the patients was 64.76 years (range, 41-83 years), and 74.4% were older than 60 years of age. Most patients were males (93.9%) and former or current smokers (84.4%). pT1b (36.1%) and pT3 (43.3%) diseases were common, and lymph node metastasis was observed in 48.9% of patients. Tumors were frequently localized in the lower esophagus (65.5%).

FGFR1 and MYC alterations in ESCC and the associated clinicopathological features

In ESCCs, *FGFR1* amplification was detected in 21.4% (37/173) of patients (high amplification in 19.7%, n = 34 and low amplification in 1.7%, n = 3; Figure 1A and B). *MYC* amplification was found in 54.2% (91/168) of patients (high amplification in 42.3%, n = 71 and low amplification in 11.9%, n = 20; Figure 1C and D). MYC expression was observed in 58.9% (106/180) of patients (weak expression in 30%, moderate expression in 22.8% and strong expression in 6.1%; Figure 1E and F). *MYC* amplification was positively correlated with MYC expression (P = 0.002; data not shown). *FGFR1* amplification status was not associated with *MYC* amplification or protein expression.

The relationships between *FGFR1* or MYC status and clinicopathological features are summarized in Table 2. ESCC patients with *FGFR1* amplification were younger than those without *FGFR1* amplification (mean ± SD, 62.3 ± 8.4 years versus 65.6 ± 7.4 years, P = 0.022). Other clinicopathological parameters including sex, histological differentiation, smoking status and TNM stage were not significantly correlated with

Table 2 Correlation among fibroblast growth factor receptor 1 amplification, MYC expression and clinicopathological features[1]

Variables	FGFR1, *n* (%)			MYC, *n* (%)		
	No amplification	Amplification	*P* value	No expression	Expression	*P* value
Age (yr)						
≤ 60	30/44 (68.2)	14/44 (31.8)	0.058	18/46 (39.1)	28/46 (60.9)	0.862
> 60	106/129 (82.2)	23/129 (17.8)		56/134 (41.8)	78/134 (58.2)	
Smoking						
No	21/28 (75)	7/28 (25)	0.619	12/28 (42.9)	6/28 (57.1)	0.836
Yes	115/145 (79.3)	30/145 (20.7)		61/151 (40.4)	90/151 (59.6)	
Histological grade						
WD	29/34 (85.3)	5/34 (14.7)	0.350	17/35 (48.6)	18/35 (51.4)	0.267
MD	90/117 (76.9)	27/117 (23.1)		43/119 (36.1)	76/119 (63.9)	
PD	11/16 (68.8)	5/16 (31.2)		10/18 (55.6)	8/18 (44.4)	
Others	6/6 (100)	0/6 (0)		4/8 (50)	4/8 (50)	
Localization						
Upper	6/7 (85.7)	1/7 (14.3)	0.981	4/7 (57.1)	3/7 (42.9)	0.688
Middle	34/42 (81)	8/42 (19)		20/44 (45.5)	24/44 (54.5)	
Lower	86/111 (77.5)	25/111 (22.5)		45/116 (38.8)	71/116 (61.2)	
EGJ	8/10 (80)	2/10 (20)		4/10 (40)	6/10 (60)	
T						
1	66/80 (82.5)	14/80 (17.5)	0.602	22/81 (27.2)	59/81 (72.8)	< 0.001
2	12/16 (75)	4/16 (25)		6/17 (35.3)	11/17 (64.7)	
3	55/73 (75.3)	18/73 (24.7)		43/78 (55.1)	35/78 (44.9)	
4	3/4 (75)	1/4 (25)		3/4 (75)	1/4 (24)	
N						
0	73/90 (81.1)	17/90 (18.9)	0.460	30/92 (32.6)	62/92 (67.4)	0.023
1-3	63/83 (75.9)	20/83 (24.1)		44/88 (50)	44/88 (50)	
Stage						
Ⅰ	51/62 (82.3)	11/62 (17.7)	0.694	18/63 (28.6)	45/64 (71.4)	< 0.001
Ⅱ	40/52 (76.9)	12/52 (23.1)		17/53 (32.1)	36/53 (67.9)	
Ⅲ	45/59 (76.3)	14/59 (23.7)		39/64 (60.9)	25/64 (39.1)	

[1]Differences in the proportions of fibroblast growth factor receptor 1 (*FGFR1*) amplification and MYC expression according to the clinicopathological variables were compared using Fisher's exact test. WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; EGJ: Esophagogastric junction.

Table 3 Correlation of fibroblast growth factor receptor 1 and MYC amplification status between the tumors of the primary and the metastatic lymph nodes, *n* (%)

		Metastatic lymph nodes		Total	*P* value
		No amplification	Amplification		
FGFR1 Primary tumor	No amplification	42 (91.3)	3 (6.7)	45 (100)	< 0.001
	Amplification	4 (36.4)	7 (63.6)	11 (100)	
	Total	46 (82.1)	10 (17.9)	56 (100)	
MYC Primary tumor	No amplification	17 (63.0)	10 (37.0)	27 (100)	1.000
	Amplification	12 (60.0)	8 (40.0)	20 (100)	
	Total	29 (61.7)	18 (38.3)	47 (100)	

FGFR1: Fibroblast growth factor receptor 1.

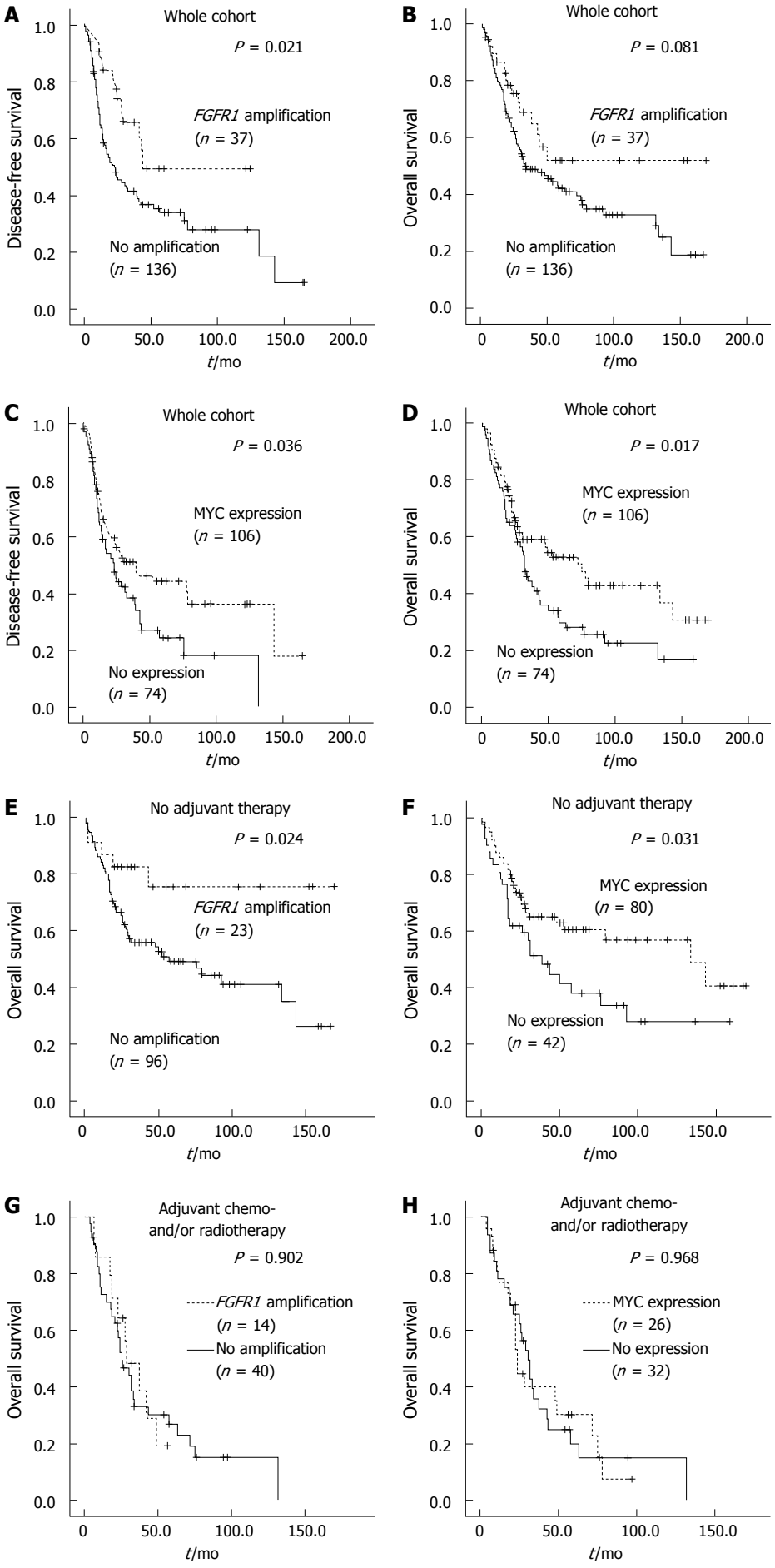
FGFR1 amplification. In contrast, MYC expression was higher in patients with early pT stage disease (P < 0.001), without lymph node metastasis (P = 0.023) or with early TNM stage disease (P < 0.001). In contrast, *MYC* amplification was not significantly correlated with clinicopathological features.

Comparison of FGFR1 amplification status between primary and metastatic lesions in regional lymph nodes

FGFR1 amplification was evaluated in matched primary tumors and metastatic lymph nodes of 56 patients, and a significantly positive correlation was found (P < 0.001; Table 3). Briefly, *FGFR1* amplification in the primary tumor was observed in 11 of 56 cases, and 7 (63.6%) patients also showed *FGFR1* amplification in metastatic tumors of the regional lymph nodes. In contrast, only 3 (6.7%) of the 45 cases who did not have *FGFR1* amplification in the primary tumor exhibited *FGFR*1 amplification in metastatic tumors of the lymph nodes. In contrast, *MYC* gene copy status was not correlated with the primary tumors or metastatic tumors of the lymph nodes (Table 3).

Prognostic significance of FGFR1 and MYC status in patients with ESCC

The mean and median follow-up times of 180 patients were 43.2 and 29.8 mo (range, 0.6-169.4 mo). The 5-year DFS and OS rates for all patients were 24% and 26%, respectively, depending on the stage as follows: 40.6% and 44.7% in stage Ⅰ, 22.9% and 27.6% in stage Ⅱ and 5.0% and 7.7% in stage Ⅲ, respectively. Kaplan-Meier analysis revealed that DFS of ESCC patients with *FGFR1* amplification was significantly prolonged compared with those without *FGFR1* amplification (P = 0.021; Figure 2A). OS also tended to be longer in patients with *FGFR1* amplification compared with those without *FGFR1* amplification (P = 0.081; Figure 2B). ESCC patients with *MYC*


A
Whole cohort
P = 0.021
FGFR1 amplification
(n = 37)
No amplification
(n = 136)
Disease-free survival
t/mo
B
Whole cohort
P = 0.081
FGFR1 amplification
(n = 37)
No amplification
(n = 136)
Overall survival
t/mo
C
Whole cohort
P = 0.036
MYC expression
(n = 106)
No expression
(n = 74)
Disease-free survival
t/mo
D
Whole cohort
P = 0.017
MYC expression
(n = 106)
No expression
(n = 74)
Overall survival
t/mo
E
No adjuvant therapy
P = 0.024
FGFR1 amplification
(n = 23)
No amplification
(n = 96)
Overall survival
t/mo
F
No adjuvant therapy
P = 0.031
MYC expression
(n = 80)
No expression
(n = 42)
Overall survival
t/mo
G
Adjuvant chemo-
and/or radiotherapy
P = 0.902
FGFR1 amplification
(n = 14)
No amplification
(n = 40)
Overall survival
t/mo
H
Adjuvant chemo-
and/or radiotherapy
P = 0.968
MYC expression
(n = 26)
No expression
(n = 32)
Overall survival
t/mo

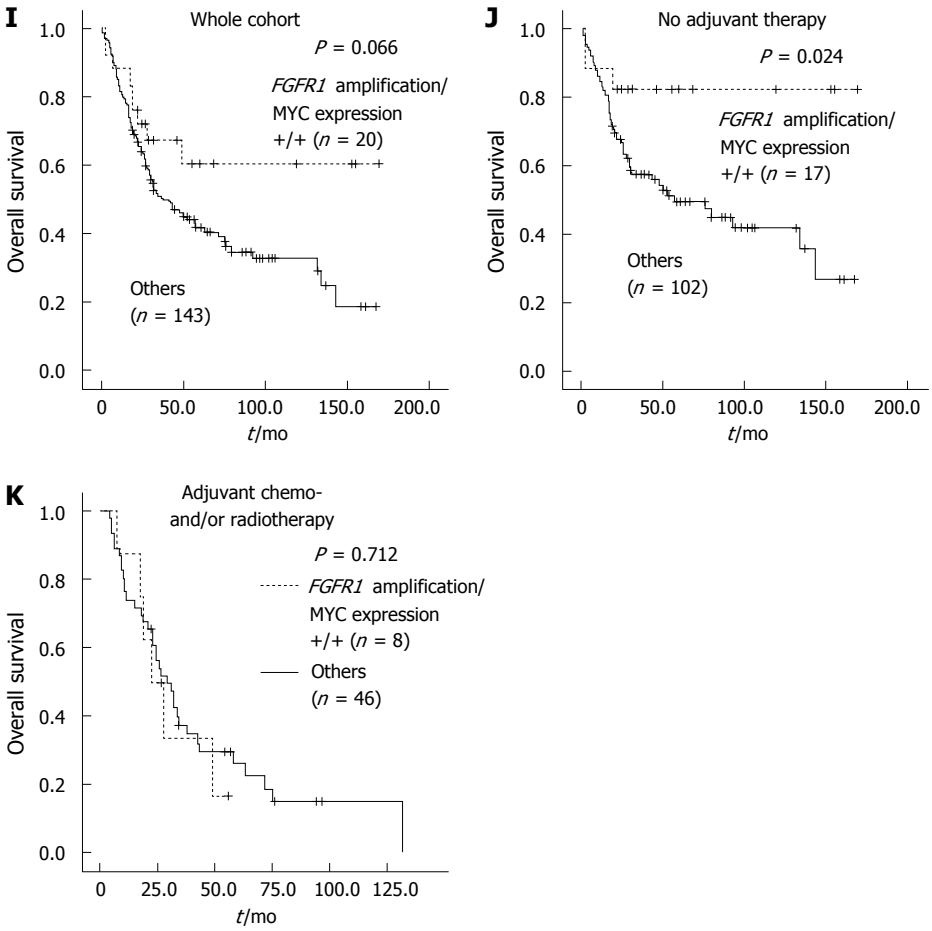


Figure 2 Kaplan-Meier plots and log rank test results. Disease-free survival (DFS) and overall survival (OS) in patients with resected esophageal squamous cell carcinoma (ESCC); A and B: According to *FGFR1* amplification; C and D: According to MYC expression status; OS was also plotted according to *FGFR1* amplification and MYC expression statuses; E and F: In patients with ESCC who did not receive adjuvant therapy; G and H: in those with ESCC who received adjuvant therapy; I-K: OS of patients with ESCC according to combined *FGFR1* amplification and MYC expression status was plotted and analyzed in all patients with resected ESCC, as well as in those with and without adjuvant therapy, respectively. *FGFR1*: Fibroblast growth factor receptor 1.

amplification tended to have a longer DFS and OS than did those without *MYC* amplification, but statistically insignificant (P = 0.064 and 0.423, respectively; data not shown). However, patients with MYC expression had a significantly longer DFS (P = 0.036) and OS (P = 0.017) compared with those without MYC expression (Figure 2C and D).

Prognostic significance of FGFR1 amplification and MYC expression according to adjuvant chemo- and/or radiotherapy status in patients with ESCC

Adjuvant chemo- and/or radiotherapy after surgical tumor resection was performed in 58 (32.2%) patients with ESCC. The mean OS of 112 patients who did not receive adjuvant chemo- and/or radiotherapy was 89.5 mo, which was significantly better than the OS of patients who received adjuvant therapy (42.5 mo). The patients who received adjuvant treatment showed a higher pT, nodal metastasis and a higher stage (all P < 0.001). These data suggest that patients given adjuvant therapy have unfavorable clinical features and aggressive biological behavior, leading to adjuvant therapy. Thus, we performed survival analysis in patients with and without adjuvant therapy separately. In patients without adjuvant therapy, *FGFR1* amplification and MYC expression were significantly associated with prolonged OS (P = 0.024 and 0.031, respectively; Figure 2E and F), but not in patients who received adjuvant chemo- and/or radiotherapy (Figure 2G and H).

Multivariate Cox analysis for OS incorporating age, T stage, lymph node metastasis, *FGFR1* amplification and MYC expression revealed that age, T and N stage were independent poor prognostic factors in all patients as well as in both groups of patients with and without adjuvant therapy (Table 4). In contrast, *FGFR1* amplification was found to be an independent favorable prognostic factor in all patients (HR = 0.532 with 95%CI: 0.302-0.937, P = 0.029) and in patients without adjuvant therapy (HR = 0.301 with 95%CI: 0.117-0.774, P = 0.013), but not in patients with adjuvant therapy (Table 4). MYC expression lost its prognostic significance in multivariate Cox analysis (Table 4).

Table 4 Multivariate analysis for overall survival in patients with esophageal squamous cell carcinoma

Variables	Category	Whole cohort		No adjuvant chemo- and/or radiotherapy		Adjuvant chemo- and/or radiotherapy	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (yr)	≤ 60 *vs* > 60	1.805 (1.102-2.955)	0.019	2.371 (1.088- 5.167)	0.030	1.81 (0.896-3.656)	0.098
T	1, 2, 3, 4	-	0.005	-	0.091	-	0.009
	1 *vs* 2	1.204 (0.523-2.773)	0.663	1.589 (0.591-4.269)	0.358	1.260 (0.251-6.324)	0.779
	1 *vs* 3	2.373 (1.454-3.872)	0.001	2.115 (1.126-3.973)	0.020	4.136 (1.691-10.119)	0.002
	1 *vs* 4	1.902 (0.550-6.575)	0.310	0.632 (0.078-5.130)	0.667	4.256 (0.820-22.092)	0.085
N	0 *vs* 1-3	1.981 (1.275-3.077)	0.002	2.351 (1.338-4.133)	0.003	0.319 (0.125-0.814)	0.017
FGFR1 amplification	None *vs* Amplification	0.532 (0.302-0.937)	0.029	0.301 (0.117-0.774)	0.013	0.830 (0.386-1.783)	0.633
MYC expression	None *vs* Expression	0.993 (0.636-1.550)	0.975	0.873 (0.478-1.595)	0.659	1.566 (0.811-3.024)	0.181

Prognostic significance of combined FGFR1 amplification and MYC expression status in patients with ESCC

ESCC patients (25/173) with both *FGFR1* amplification and MYC expression (hereafter referred to as combined positivity) exhibited prolonged DFS (P = 0.023; data not shown) and OS (P = 0.066) in Kaplan-Meier analysis (Figure 2I). Combined positivity was significantly associated with longer OS (P = 0.024) in patients who did not receive adjuvant therapy, but not in patients who received adjuvant therapy (P = 0.712; Figure 2J and K). Combined positivity was also shown to be an independent favorable prognostic factor among patients who did not receive adjuvant therapy; this was determined when multivariate Cox analysis for OS was performed and incorporated age, T stage, lymph node metastasis, and combined positivity (HR = 0.275 with 95%CI: 0.083-0.97, P = 0.034; data not shown).

DISCUSSION

In this study, we comprehensively investigated *FGFR1* amplification and *MYC* amplification and expression in ESCC to elucidate the associated clinicopathological characteristics and explore the potential of FGFR1 and MYC as targets for cancer therapy.

Several previous studies reported the prognostic implication of *FGFR1* amplification in ESCC, but the results were controversial[11,12]. *FGFR1* amplification was associated with poor prognosis or had no prognostic significance in ESCC; however, the FISH criteria for *FGFR1* amplification were not identical[11,12]. In the present study, *FGFR1* amplification was a favorable prognostic indicator in patients with resected ESCC, which was in conflict with a previous report using the same FISH criteria[11]. In a study using FISH and different criteria, *FGFR1* amplification was not associated with clinical outcomes in patients with ESCC[12]. Similarly, in the case of pulmonary SCC, the prognostic implication of *FGFR1* amplification was controversial[19-21]. One study demonstrated that *FGFR1* amplification was an independent favorable prognostic factor in pulmonary SCC and large cell carcinoma[19], which contrasted with another study showing that *FGFR1* amplification was an independent negative prognostic factor in resected pulmonary SCC[20]. Consequently, a recent meta-analysis concluded that *FGFR1* amplification had no influence on the survival of patients with pulmonary SCC[22]. Notably, in this study, the association of *FGFR1* amplification with clinical outcome of resected ESCC patients was dependent on the status of adjuvant therapy; *i.e.*, *FGFR1* amplification was a favorable prognostic factor in patients with ESCC who did not receive adjuvant therapy. Adjuvant therapy after surgery for patients with stage Ⅲ-Ⅳ or lymph node metastasis prolonged survival compared with surgery alone in ESCC[23]. Therefore, adjuvant chemotherapy with or without radiotherapy is increasingly used for the treatment of advanced ESCC, although no definite criteria or regimen for adjuvant therapy has been established in ESCC. In this study, patients with adjuvant chemo- and/or radiotherapy tended to be in the advanced stage compared with those with no adjuvant therapy. Thus, FGFR1 might play variable biological roles during the progression of cancer and thereby have different prognostic significance depending on the stage and subsequent adjuvant therapy status of patients. Otherwise, it could be possible that ESCC with *FGFR1* amplification represents a biologically less aggressive group among ESCCs having variable genetic alterations. This could result in the prolonged survival of patients receiving no adjuvant therapy. FGFR1 could affect the efficacy of chemo- or radiotherapy in patients with ESCC, and thus be differently associated with the prognosis in those receiving adjuvant therapy.

To the best of our knowledge, this is the largest study to evaluate *MYC* status using IHC and FISH in ESCC. Kaplan-Meier analysis demonstrated that MYC expression, but not amplification, was associated with prolonged survival. This result might be contradictory to the role of MYC as an oncogene. In this study, MYC expression was more common in ESCC patients of younger age and in the early TNM stage, and it was not an independent prognostic factor. Thus, the favorable prognosis of patients with ESCC who showed combined *FGFR1* amplification and MYC expression in the group without adjuvant therapy might be due to the association of *FGFR1* amplification with prognosis. Based on this study, MYC status might have little, if any, prognostic implication in patients with ESCC.

However, more studies using a large cohort of patients are needed to validate the prognostic significance of MYC in ESCC.

However, this study had some limitations. First, it was a retrospective study and, as such, the specific regimen of adjuvant therapy may not have been well-controlled. Second, we used a TMA of 2 mm diameter, which may not reflect the intratumoral heterogeneity of FGFR1 and MYC status. However, comparative analysis showed that FGFR1 amplification status was not significantly different between primary and nodal metastatic tumors. In contrast, MYC amplification status was significantly different between these two groups. Third, small groups were compared as a result of subgroup analysis according to the different treatment modalities. Thus, another study using large prospective cohorts is required to validate the prognostic role of FGFR1 amplification in ESCC according to adjuvant therapy status.

Although ESCC is an aggressive cancer with poor clinical outcomes, treatment approaches remain limited, requiring the development of novel strategies including targeted molecular therapy. This study demonstrated that *FGFR1* was amplified in approximately 20% of ESCCs, and moreover, *FGFR1* amplification status was maintained during lymph node metastasis; hence, this group may benefit from therapeutic inhibition of *FGFR1*. *FGFR1* amplification is considered an adequate factor to predict sensitivity to FGFR inhibitors[24]. However, FGFR inhibitors resulted in insufficient clinical responses in patients with *FGFR1*-amplified lung cancer[25,26]. A recent study showed that MYC expression might modulate the sensitivity of *FGFR1*-amplified pulmonary SCC to FGFR1 inhibitors[13]. In that study, 40% of *FGFR1*-amplified pulmonary SCCs expressed high levels of MYC[13], which was similar to our results in that 54.1% (20/37) of *FGFR1*-amplified ESCCs expressed MYC. Among the all patients with resected ESCC, 12.3% (20/163) exhibited both *FGFR1* amplification and MYC expression. Based on the pulmonary SCC study, this population could be a potential candidate for FGFR inhibitor therapy in ESCC patients. The role of therapy targeting *FGFR1* or *MYC* in ESCC remains to be explored by further *in vitro* and clinical studies.

In conclusion, *FGFR1* amplifications were observed in 21.4% of patients and combined *FGFR1* amplification and MYC expression was observed in 12.3% of patients with resected ESCC. *FGFR1* amplification had prognostic implications in patients with resected ESCC with respect to adjuvant therapy. The role of targeted therapy against FGFR1 or MYC in ESCC remains to be explored.

COMMENTS

Background

It has been demonstrated that fibroblast growth factor receptor 1 (*FGFR1*) and *MYC* are frequently co-amplified and play a role in neoplastic transformation in pulmonary squamous cell carcinoma (SCC). Moreover, a potential role of MYC as a predictor of the sensitivity to FGFR inhibitors in pulmonary SCC has been reported. Although *FGFR1* and *MYC* alterations have been reported by genomic studies for esophageal squamous cell carcinoma (ESCC), the prevalence of *FGFR1* and *MYC* alterations and their relationship remains to be clarified in patients with ESCC. Thus, we investigated *FGFR1* amplification and *MYC* amplification and expression in patients with ESCC and analyzed their clinicopathological features and prognostic significance.

Research frontiers

ESCC is one of the leading causes of cancer-related mortality worldwide and novel treatment strategies other than surgery and conventional chemo- and radio-therapy are required to improve clinical outcome. However, molecular targeted therapy for ESCC remains to be established. The results of this study contribute to clarifying the biological role of FGFR1 and MYC, and therapeutic potential of FGFR targeted therapy in patients with ESCC.

Innovations and breakthroughs

In this study, *FGFR1* amplifications were observed in 21.4% of patients and combined *FGFR1* amplification and MYC expression was observed in 12.3% of patients with resected ESCC. *FGFR1* amplification had prognostic implications in patients with resected ESCC with respect to adjuvant therapy. The role of FGFR1-targeted therapy in ESCC remains to be explored.

Applications

This study suggests that patients with ESCC harboring combined *FGFR1* amplification and MYC expression might benefit from therapies targeting FGFR1 and/or MYC, especially those with advanced disease requiring adjuvant therapies.

Terminology

FGFR1 is a receptor tyrosine kinase playing an oncogenic role in many cancers and can be targeted for molecular therapy. MYC is an oncogene and contributes to sensitivity to FGFR inhibitor in pulmonary squamous cell carcinoma (SCC). Fluorescence *in situ* hybridization (FISH) is a tool useful to evaluate the gene amplification using tumor tissues from patients with solid tumor.

Peer-review

It is a very interesting article presenting novel data on role of FGFR1 and MYC status in ESCC. All parts of the manuscript were composed correctly and they contain suitable information. Tables and figures were constructed appropriately. Statistical analysis of data was performed correctly with using the appropriate tests. All references are actual and relevant to the text of article.

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ORIGINAL ARTICLE

Retrospective Study

IFIT1 polymorphisms predict interferon-α treatment efficiency for hepatitis B virus infection

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Abstract

AIM

To investigate the association between interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) polymorphisms and interferon-α (IFNα) treatment efficiency among Chinese hepatitis B virus (HBV) infection patients.

METHODS

Two hundred and twenty five newly diagnosed chronic

hepatitis B (CHB) patients were enrolled in the study. All of these patients received IFNα treatment for a course of 48 wk, and were followed up for 24 wk after the treatment was end. Clinical information about virological response, hepatitis B e antigen (HBeAg) seroconversion rate and combined response at the end of the treatment, as well as the sustained response by the time of following up 24 wk after the treatment, was collected. Four tag-single nucleotide polymorphisms (SNPs) of *IFIT1* were selected and assessed for their association with these clinical outcomes.

RESULTS

At the end of the treatment, HBeAg seroconversion was observed in 27.1% patients. Thirty-six point nine percent patients achieved virological response, and 15.6% patients exhibited combined response. Sustained response was obtained in 26.2% patients. The main HBV genotype of the study was genotype B. Patients who infected with HBV genotype B or C showed better treatment efficiency, no matter which clinical outcome was considered. Among the four SNPs assessed, rs303218 (A > G) was found to be significantly associated with the end point virological response when assuming additive model [OR = 0.64 (95%CI: 0.42-0.96), *P* = 0.032]. Patients who carried rs303218 GG genotype had a rather higher rate of achieving virological response (response rate: 52%, OR = 0.40, 95%CI: 0.18-0.91; *P* = 0.028) when compared to those had AA genotype (response rate: 27%). The most significant interaction was observed in patients who had relative lower baseline aspartate transaminase. No association between SNPs and HBeAg seroconversion, combined response or sustained response was observed.

CONCLUSION

IFIT1 involves in the regulation of IFNα treatment for CHB and its polymorphism rs303218 can predict the end point virological response. The finding requires further validation.

Key words: Virological response; Hepatitis B virus infection; *IFIT1*; Interferon-α therapy; Polymorphism

Core tip: Interferon-α (IFNα) is the first line treatment for chronic hepatitis B virus (HBV) infection (CHB). However, its efficiency differs and biomarkers for predicting responses of IFNα are needed. The current study performed an epidemiologic study to investigate the association between Interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) polymorphisms and clinical responses of IFNα treatment in newly diagnosed chronic HBV infection patients among Chinese population. We identified that *IFIT1* polymorphism rs303218 could be a predictor for the end point virological response of IFNα therapy. The finding may provide insight to the potential role of IFIT1 in the individualized treatment of CHB in the future.

Xie DY, Wang SM, Yang JM, Wang LH, Chen HY, Huai C, Shang J, Mao Q, Lei CL, Luo GH, Qian J, Lu DR. *IFIT1* polymorphisms predict interferon-α treatment efficiency for hepatitis B virus infection. *World J Gastroenterol* 2016; 22(44): 9813-9821 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9813.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9813

INTRODUCTION

Hepatitis B infection is a life-threatening disease caused by hepatitis B virus (HBV), which attacks liver. An estimated 350 million people are infected with hepatitis B chronically worldwide, which makes it a major global health problem[1]. According to WHO, more than 780000 people die of cirrhosis and hepatocellular carcinoma (HCC) caused by chronic hepatitis B (CHB). Currently, immune modulators such as interferon-α (IFNα) or pegylated interferon-α (PEG-IFNα) and antiviral agents such as nucleotide analogues (NAs) are two approved treatments for CHB patients[2]. Compared with NAs treatment, IFNα is less likely to develop drug resistance and its finite duration is an attractive treatment strategy for CHB patients. IFNα treatment showed high rates of off-therapy host immune control over HBV and increased rates of hepatitis B e antigen (HBeAg)/hepatitis B surface antigen (HBsAg) loss or seroconversion over time[3]. However, the fact that only 30%-40% patients benefit from the IFNα therapy is still an obstacle in CHB management[4]. Therefore, it is necessary to discover predictors for outcomes of IFNα treatment to improve the personalized therapy for CHB patients. Several host and virus factors such as gender, serum HBV DNA level and alanine aminotransferase (ALT) level are considered to have influence on IFNα efficiency, but they are weak at predicting responses at individual level[5]. More and more researches have shown that host genetic factors may play an important role in the response to IFNα treatment. Single nucleotide polymorphisms (SNPs) located on *IL28B* are reported to affect the response to IFNα based therapy for CHB patients[6-8]. Polymorphisms on *HLA-DP* and *IRF5* are also associated with IFNα treatment efficiency[9,10]. It is also reported that genetic variants on *STAT4* influenced the response of IFNα among CHB patients[11]. All the evidences indicate genetic variations on genes involved in immune response or IFNα signaling may lead to different clinical outcomes of IFNα therapy.

After virus infection, the expression of virus-responsive genes and antiviral cytokines such as type I interferon are induced to limit virus replication and modulate adaptive immune response. Interferon-stimulated genes (ISGs) are a subset of genes response to RNA- or DNA- virus infection or type Ⅰ IFN treatment,

and they are mainly induced by IFN-α/β[12]. Under basal condition, ISGs are not expressed in most cell types. But they can be induced immediately to a high level after virus infection or IFN treatment[13]. Their products take on diverse roles such as enhancing innate immune capabilities, inhibiting virus infection and negatively regulating signaling through the JAK-STAT pathway[14]. Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) which is an effector molecule in antivirus pathways, locates in the cytoplasm. It is an important member of ISGs family which lacking enzymatic domains or activity. Tetratricopeptide repeats (TPR) motif mediates protein-protein interaction. Proteins containing TPR motifs regulate cell cycle, transcription, protein transport and protein folding, which enable IFIT1 to serve as an effector molecule on virus replication during responses to viral infections[15]. IFIT1 is induced within two hours of exogenous IFN-α treatment[16]. It is reported that IFIT1 acts as an important innate immune bottleneck which shows positive regulation on downstream genes[17]. High level of type Ⅰ IFN is observed in IFIT1-expressing cells[18]. Researches have indicated that the antiviral activity of IFIT1 is modulated by 2′-O methylation of viral RNA. Abrogation of 2′-O methylation resultes in enhanced type Ⅰ IFN and IFIT1 sensitivity[19-21]. IFIT1 can sense the methylation state of capped RNA and inhibit viruses by binding to their 5′ cap structure that lack 2′-O methylation[22,23]. It can also sense viral RNA by recognizing uncapped 5′-ppp and stop it from actively replicating[24]. Despite sensing 2′-O methylation viral RNA, IFIT1 also exerts its antivirus function through inhibiting steps in translation initiation. It is reported that IFIT1 reduces the translation efficiency by binding to the subunits of eIF3 complex, which functions in several steps in translation initiation[25,26]. IFIT1 is also responsible for IFN-induced alteration of virus transcription and protein synthesis[27]. Silencing of IFIT1 leads to remarkable increased HBV replication, which indicates that IFIT1 plays an important role in the regulation of HBV transcription and posttranscriptional procedure[28]. All the evidences indicate that IFIT1 is an important effector in both virus infection and IFNα treatment. It has been reported that IFIT1 acts as a potential biomarker for Peg-IFNα treatment efficiency in hepatitis C virus (HCV) patients[29]. However, few researches mention about IFIT1's role in HBV infected patients who treated with IFNα. To illuminate whether IFIT1 related to IFNα treatment efficiency for CHB, we conducted an association study that assessed the relationship between tag-SNPs on IFIT1 and clinical outcomes of IFNα treatment among 225 Chinese CHB patients.

MATERIALS AND METHODS

Patient recruitment

Patients enrolled in this study were newly diagnosed HBeAg-positive CHB patients who were recruited from nine Chinese hospital between August 2009 and May 2012, including the Third Affiliated Hospital of Sun Yat-Sen University, the Eighth People's Hospital of Guangzhou, Nanfang Hospital, Shenzhen Third People's Hospital, the First Affiliated Hospital of Guangxi Medical University, Henan Provincial People's Hospital, the First Affiliated Hospital of Third Military Medical University, Xiangya Hospital Central South University, and Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology. The major criteria for clinical data collection and patient recruitment were: (1) written informed consent available and adherence to the treatment schedule; (2) age 18-60 years old; (3) HBsAg-positive and HBeAg-positive for more than six months; (4) serum level of HBV DNA > 20000 IU/mL; (5) serum ALT level > 40 IU/L; and (6) no diagnosed HCC or suspected to have HCC. The main exclusion criteria were: (1) previous IFN treatment, nucleos(t)ide analogues treatment or immunomodulatory therapy within six months; (2) coinfection with HCV, hepatitis D virus or human immunodeficiency virus (HIV); (3) autoimmune hepatitis, steatohepatitis or other active hepatopathy; and (4) evidence of decompensated liver disease. All patients received antivirus therapy with 6 MU IFNα-2b (rHuIFNα-2b, Amoytop) every other day for 48 wk. Evaluation of therapeutic efficiency was performed at the end of the treatment course and by the time of following up to 24 wk. Efficiency of the treatment was assessed by end point response including HBeAg seroconversion, virologic response and combined response at 48 wk, and by sustained response which was assessed at 72 wk. HBeAg seroconversion was defined as the loss of HBeAg and the presence of anti-HBe. Virological response was defined as serum HBV DNA level < 2000 IU/L. Combined response was defined as the combination of HBeAg seroconversion and virological response, as well as the normalization of serum ALT. Sustained response was defined as the combined response at week 72 after the first dose of the treatment.

SNP selection and genotyping

IFIT1 is about 13.9 kb long and locates on chromosome 10q23.31. To investigate the association between IFIT1 and IFNα treatment efficiency, 4 tag-SNPs located in *IFIT1* gene region were selected according to the genotype data of Han Chinese in Beijing (CHB) population from the phase II HapMap SNP database, by software Haploview 4.1 (available at http://www.broadinstitute.org/haploview). The thresholds for tag-SNP selection were defined as 0.8 for correlation coefficient and a cutoff of 0.2 for MAF. Blood sample were collected at the time of recruitment. Genome DNA was extracted by salt-out method. All the selected SNPs were genotyped using DNA sequencing on illumine Miseq high throughout sequencing platform. Random duplicate sample were performed and all the

Table 1 Patient charateristics

Patient characteristic	Total patient (*n*)
Total patient	225
Gender	225
Male	163 (72.4)
Female	62 (27.6)
Age	225
Median age (range)	26 (18-56)
Baseline ALT	225
Median (range)	150 (70-359)
Baseline AST	225
Median (range)	83 (30-294)
Baseline HBV DNA copies (log_{10}IU/L)	225
Median (range)	7.36 (3.52-8.90)
HBV genotype	197
Type A	8 (4.1)
Type B	94 (47.7)
Type C	15 (7.6)
Type B + C	80 (40.6)
Virological response[1]	225
Response	83 (36.9)
Non-response	142 (63.1)
HBeAg seroconversion[2]	225
Response	61 (27.1)
Non-response	164 (72.9)
Combined response[3]	225
Response	35 (15.6)
Non-response	190 (84.4)
Sustained response[4]	225
Response	59 (26.2)
Non-response	166 (73.8)

[1]Virological response was defined as serum HBV DNA level < 2000 IU/L at the end of the treatment; [2]HBeAg seroconversion was defined as the loss of HBeAg and the presence of anti-HBe at the end of the treatment; [3]Combined response was defined as the combination of HBeAg seroconversion and virological response, as well as the normalization of serum ALT at the end of the treatment; [4]Sustained response was defined as the combined response at week 72 after the first dose of the treatment. ALT: Alanine aminotransferase; AST: Aspartate transaminase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

samples were concordant with the genotyping results. All SNPs were in Hardy-Weinberg equilibrium (P > 0.001). More details about these four tag-SNPs were shown in Supplementary Table 1.

Statistical analysis

Patient characteristics and clinical variables were test by χ^2 tests or Student's t test against treatment responses. Factors that had P value < 0.05 were regarded as covariates (Table 2). Univariable logistic regression analysis was performed to assess allele frequency distribution of *IFIT1* SNPs in different patient groups. Chi-square test and unconditional logistic regression adjusted for covariates were used to assess whether *IFIT1* SNPs' genotypes had statistically significant difference in the distribution of clinical outcomes, and to estimate the association between efficiencies and SNPs by OR and in additive, dominant, recessive or co-dominant model. Stratified analysis was performed to investigate significant SNPs' effects in different subgroups. All P values reported in this study were two-sided, and P < 0.05 was considered statistically significant. All the statistical analyses were performed by SPSS (version 15.0; SPSS Inc., Chicago, Ill).

RESULTS

Patient characteristics and clinical outcomes

225 CHB patients who met the recruitment criterion were included in the study to investigate the association between *IFIT1* polymorphisms and IFNα treatment responses. Table 1 summarized patient characteristics and clinical outcomes. Male patients accounted for 72.4% of this cohort and the median age was 26 years old. The median baseline ALT and aspartate transaminase (AST) were 150 IU/L and 83 IU/L, respectively. Baseline HBV DNA level's median is 7.36 log10 IU/mL. The main HBV genotype of this cohort was genotype B, which took over 47.7% of all the patients. Forty point six percent patients infected with both genotype B and C HBV at the same time. After 48 wk IFNα treatment, 27.1% patients achieved HBeAg seroconversion. 36.9% patients' serum HBV DNA level decreased below 2000 IU/L which meant virological response were attained in these patients. Combined response was observed in 15.6% patients. Twenty-four weeks off treatment follow-up showed that 26.2% patients obtained sustained response. According to Table 2, HBV genotype had impact on IFNα therapy responses. Patients who infected with HBV genotype B or C showed better treatment efficiency than those infected with genotype A or those infected with both genotype B and C, no matter which clinical outcome was considered. As shown in Table 3, the level of baseline ALT was associated with HBeAg seroconversion (P = 0.020), and the distribution of combined response was significantly different between male and female patients (P = 0.027). HBV genotype exhibited significant association with all the clinical outcomes including virological response (P = 0.012), HBeAg seroconversion (P = 0.001), combined response (P = 0.038) and sustained response ($P = 2.37 \times 10^{-4}$). Other clinical characteristics including age, baseline AST and baseline HBV DNA level, showed association with none of the clinical outcomes.

IFIT1 polymorphisms and IFNα treatment's virological response

All the SNPs were evaluated for their association with IFNα treatment efficiency, including the end point responses and sustained response. It was considerable that none of the *IFIT1* SNPs' allele frequency showed distribution differences in HBeAg seroconversion, combined response or sustained response (data not shown), but all of them had significantly different allele frequency distribution among patients who achieved virological response (Table 4). So we further assessed these SNPs' association with virological response

Table 2 Hepatitis B virus genotype and interferon-α treatment responses

HBV genotype	Total	Virological response[1]		HBeAg seroconversion[2]		Combined response[3]		Sustained response[4]	
		R	%	R	%	R	%	R	%
Type A	8	3	37.5	1	12.5	1	12.5	1	12.5
Type B	94	42	44.7	38	40.4	19	20.2	37	39.4
Type C	15	10	66.7	5	33.3	5	33.3	6	40.0
Type B + C	80	22	27.5	11	13.8	7	8.8	10	12.5

[1]Virological response was defined as serum HBV DNA level < 2000 IU/L at the end of the treatment; [2]HBeAg seroconversion was defined as the loss of HBeAg and the presence of anti-HBe at the end of the treatment; [3]Combined response was defined as the combination of HBeAg seroconversion and virological response, as well as the normalization of serum ALT at the end of the treatment; [4]Sustained response was defined as the combined response at week 72 after the first dose of the treatment. HBV: Hepatitis B virus; R: Response; HBeAg: Hepatitis B e antigen.

Table 3 Association between clinical factors and responses

Patient characteristics	Virological response			HBeAg seroconversion			Combined response			Sustained response		
	R	NR	*P* value[1]	R	NR	*P* value[1]	R	NR	*P* value[1]	R	NR	*P* value[1]
Gender			0.113			0.081			0.027[2]			0.204
Male	55	108		39	163		20	163		39	163	
Female	28	34		22	62		15	62		20	63	
Age			0.151			0.723			0.924			0.116
Baseline ALT			0.070			0.020[2]			0.391			0.181
Baseline AST			0.205			0.113			0.443			0.279
Baseline HBV DNA level			0.121			0.554			0.617			0.217
HBV genotype			0.012[2]			0.001[2]			0.038[2]			2.37×10^{-4}[2]
Type A	3	5		1	7		1	7		1	7	
Type B	42	52		38	56		19	75		37	57	
Type C	10	5		5	10		5	10		6	9	
Type B + C	22	58		11	69		7	73		10	70	

[1]*P* value was calculated by χ^2 test or Student's *t*-test depending on which variables were analyzed; [2]$P < 0.05$. ALT: Alanine aminotransferase; AST: Aspartate transaminase; HBV: Hepatitis B virus; R: Response; NR: Non-response; HBeAg: Hepatitis B e antigen.

Table 4 The association between single nucleotide polymorphisms and virological response by allele frequencies

SNP ID	Genotype	No./total[1]	%	*P*[2]	OR	95%CI
rs303218	A	75/242	0.31	0.005[3]	1 (Reference)	
	G	87/198	0.44		0.57	0.39-0.85
rs303215	T	93/227	0.41	0.045[3]	1 (Reference)	
	C	69/217	0.32		1.49	1.01-2.20
rs11203109	T	109/341	0.32	0.020[3]	1 (Reference)	
	C	37/81	0.46		0.56	0.34-0.92
rs303212	C	93/284	0.33	0.022[3]	1 (Reference)	
	T	65/148	0.44		0.62	0.41-0.94

[1]Number indicated the patients who responsed in the same group; [2]*P* value was calculated by univariable logistic regression analysis; [3]$P < 0.05$. SNP: Single nucleotide polymorphism.

by genotype. χ^2 test showed that the distribution of rs303218 (A > G) genotype was significant different ($P = 0.022$), which is the most significant SNP from the allele frequency distribution assessment. Patients who carried rs303218 GG genotype had a rather high rate of virological response (response rate: 52%) when compared to patients who had AA genotype (response rate: 27%), with OR of 0.40 (95%CI: 0.18-0.91) and *P* value of 0.028. Unconditional logistic regression adjusted by covariate, which was HBV genotype for virological response, showed that rs303218 presented a protective role in IFNα virological response when assuming additive model (OR = 0.64; 95%CI: 0.42-0.96; $P = 0.032$) (Table 5). Then stratified analysis was performed to investigate the SNP rs303218's effect in different subgroups. Continuous variables such as age, baseline ALT, baseline AST and baseline HBV DNA level, were dichotomized by median. According to Table 6, the most significant association between rs303218 and virological response was observed among patients who had baseline AST ≤ 83 (OR = 0.31; 95%CI: 0.16-0.61; $P = 0.001$). And rs303218 could also be a better virological response predictor for male patients (OR = 0.53; 95%CI: 0.33-0.88; $P = 0.013$), patients who had baseline ALT ≤ 150 (OR = 0.38; 95%CI: 0.20-0.74; $P = 0.003$)

Table 5 The association between rs303218 and virological response

SNP ID	Genotype	Genetic model[1]	No./total[2]	%	χ^2 test	Logistic regression analysis		
					P value[3]	OR	95%CI	*P* value[3,4]
rs303218	A A		19/70	0.27	0.022[3]	1 (Reference)		
	G A		37/102	0.36		0.77	0.38-1.55	0.467
	G G		25/48	0.52		0.40	0.18-0.91	0.028[3]
		Add				0.64	0.42-0.96	0.032[3]

[1]The best fitting model was shown; [2]Number indicated the patients who responsed in the same group; [3]$P < 0.05$; [4]*P* value was calculated by logistic regression analysis with adjustment of patient characteristics with $P < 0.05$ in univariate analysis (the adjusting covariate for virological response was HBV genotype). HBV: Hepatitis B virus.

Table 6 Stratified analyses of rs303218 and virological response

Variables	No./total[1]	rs303218		
		OR	95%CI	*P* value[2]
Age (yr)				
≤ 26	39/117	0.79	0.42-1.48	0.453
> 26	44/108	0.59	0.33-1.04	0.070
Gender				
Male	55/163	0.53	0.32-0.88	0.013[3]
Female	28/62	1.03	0.46-2.31	0.947
Baseline ALT				
≤ 150	38/113	0.38	0.20-0.74	0.003[3]
> 150	45/112	0.91	0.52-1.60	0.734
Baseline AST				
≤ 83	34/114	0.31	0.16-0.61	0.001[3]
> 83	49/111	1.10	0.62-1.96	0.752
Baseline HBV DNA copies (log_{10} IU/mL)				
≤ 7.36	47/113	0.86	0.49-1.52	0.611
> 7.36	36/112	0.47	0.25-0.88	0.018[3]
HBV genotype				
Type B	42/94	0.74	0.42-1.31	0.304
Type C	10/15	2.19	0.36-13.51	0.397
Type B + C	22/80	0.41	0.19-0.86	0.018[3]

[1]Number indicated the patients who responsed in the same group; [2]*P* value was calculated by logistic regression analysis with adjustment of patient characteristics with $P < 0.05$ in univariate analysis in additive model (the adjusting covariate for virological response was HBV genotype); [3]$P < 0.05$. ALT: Alanine aminotransferase; AST: Aspartate transaminase; HBV: Hepatitis B virus.

and patients whose baseline HBV DNA level were higher than 7.36 log10 IU/mL (OR = 0.47; 95%CI: 0.25-0.88; $P = 0.018$), as well as patients who infected with both HBV genotype B and C (OR = 0.41; 95%CI: 0.19-0.86; $P = 0.018$).

DISCUSSION

IFNα or PEG-IFNα is the first-line treatment for Chronic HBV infection. It can maintain high rates of off-therapy host immune control over HBV. However, IFNα therapy gives benefits to only 30%-40% CHB patients, which suggests the necessity for discovering efficiency predictors for IFN treatment to improve the personalized therapy for CHB patients. It is reported that female patients, higher ALT level and lower serum HBV DNA level may indicate a better IFNα response[30]. But all of these host or virus factors are not ideal predictors at individual level. Researchers expect that biomarkers that rely on the basis of patients' genetic background can highlight the road to personalized medicine. As one of the key components of IFNα induced pathways, IFIT1 is indispensable for IFNα to eliminate HBV. However, few researches focused on its role in HBV management, especially its pharmacogenetic effects. In this study, we investigated whether *IFIT1* gene polymorphisms could predict IFNα treatment efficiency among Chinese CHB patients. The results showed that rs303218 was associated with end point virological response after 48 wk IFNα therapy. Patients who carried GG genotype of rs303218 achieved higher rate of virological response when compared to GA/AA genotype.

IFNα is an important innate immune response cytokine which acts as the first line defense of HBV infection[31]. Patients who have CHB may have reduced ability of producing IFNα, but they show response to exogenous IFNα and then induce ISGs expression to activate related signaling to inhibit HBV replication[32,33]. The function of ISGs includes enhancing innate immune capabilities and inhibiting virus infection. IFIT1 is one of the most immediately induced ISGs after exogenous IFNα treatment. The most well-known function of IFIT1 is that it can sense and recognize the 2'-O unmethylated RNA and block the translation of viral RNA lacking 2'-O methylation[22,34]. It can also recognize the uncapped 5'-ppp and stop it from actively replicating[35]. But viruses that using cellular RNA polymerase II to synthesize their mRNA may escape the IFIT1-mediated restriction[36]. However, there are other ways that IFIT1 exerts its anti-virus function. For example, IFIT1 acts as an important modulator in virus transcription and replication. It can inhibit cap-dependent protein synthesis by binding to the subunits of translation initiation complex eIF3[25,37]. Researches has demonstrated that IFIT1 can restrict HCV growth by inhibiting HCV replication, and the expression level of IFIT1 can be potential biomarker of response to IFNα in patients with HCV[29,38]. IFIT1 restricts the translation and replication of many other viruses such as HPV, HIV and alphavirus, and promotes the induction of IFNα to enhance immune response[18,39,40]. All these evidences demonstrate

the importance of IFIT1 in immune system and virus inhibition. STAT/JAK pathway has been reported to be a vital role in pharmacogenomics of IFNα treatment. As an essential member of STAT/JAK pathway, IFIT1 may participate in affecting IFNα therapy responses by modulating downstream STAT/JAK signaling. Recently, it is reported that IFIT1 involves in the control of HBV by limiting replication and slowing down the spread of HBV, which further illustrates that IFIT1 takes part in HBV restriction and may influence IFNα treatment efficiency[28]. Our findings indicated that rs303218 was associated with virological response, which also suggested IFIT1's indispensable role in controlling HBV replication.

As we know that, the polymorphism of rs303218 locates in the intron region of *IFIT1*. Other than causing missense mutation which results in protein dysfunction, intronic polymorphisms may locate in the regulatory element sites, such as splice donor, acceptor, or cis-regulatory element region, which means they might regulate cell metabolism through modulating mRNA splicing and gene expression patterns[41-43]. NCBI database shows that IFIT1 has three isoforms. They are different from each other by their different alternate initiation translation site. A reasonable conjecture is that different IFIT1 isoform may have different affinity to IFIT family members or to the translation initiation complex such as eIF3, or they may display different capability of recognizing 2'-O unmethylated RNA or uncapped 5'-ppp. However, no evidence was found to support that variants on *IFIT1* facilitated gene splicing or gene expression. A recent research showed that a variant known as rs304478, which located within 2 kb upstream of *IFIT1*, was an independent predictive factor for pegylated-IFN therapy in HCV patients[44]. Although rs304478 was not included in the present study due to its relative lower MAF in Chinese population, it highlighted a fact that IFIT1 could be potential biomarker for IFNα treatment efficiency. Another assumption is that the real reason affecting IFIT1 mRNA stability or protein synthesis, is the effective SNPs which are in linkage disequilibrium with rs303218. Nevertheless, all these are just hypothesis that need more researches to discover the real facts of how *IFIT1* intronic SNPs influence IFNα treatment efficiency, and it is necessary to find the actual genetic variations that affect responses of IFNα.

In conclusion, the study investigated the association between *IFIT1* polymorphisms and clinical outcomes of IFNα treatment for CHB patients among Chinese population. The results provided evidences for IFIT1's part in IFNα treatment efficiency. Our study highlights the potential role of IFIT1 in predicting IFNα treatment's end point virological response, although the exact mechanism needs to further investigate. The findings needs to be validated in an independent cohort to further illuminate the effects of *IFIT1*'s variants for IFNα therapy. And it is meaningful to assess IFIT1's predicting potential in different ethnicities. Function studies are needed to explore the mechanism between IFIT1 and IFNα therapy.

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COMMENTS

Background

Hepatitis B infection is a major global health problem. As a life-threatening disease, many people die of chronic hepatitis B (CHB) related cirrhosis and hepatocellular carcinoma. Interferon-α (IFNα) treatment showed high rates of achieving off-therapy responses and low rate of developing drug resistance, which makes it the first-line treatment for CHB patients. However, the obstacle that only 30%-40% CHB patients benefit from IFNα treatment still limit the CHB management. Although several host and viral factors are considered to influence IFNα therapy efficiency, but they are not ideal at individual level. Researches have shown that host genetic characteristics such as genetic variations may provide new approaches to predict responses of IFNα based therapy, especially those genes involved in immune response or IFNα signaling. Interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) is one of the immediately induced genes by IFNα, which plays an important role in antivirus pathways. Deregulation of IFIT1 may have great influence on hepatitis B virus (HBV) transcription which indicated that IFIT1 could be potential biomarkers for predicting IFNα treatment efficiency. In this study, we perform an association study that investigated the relationship between polymorphisms on IFIT1 and clinical outcomes of IFNα therapy among Chinese CHB patients.

Research frontiers

IFIT1 is an important effector molecule in antivirus pathways. It is responsible for IFN-induced alteration of virus transcription and protein synthesis. However, few prior researches focus on IFIT1's role in CHB patients who treated with IFNα. The results of the study contribute to illustrating IFIT1's role in IFNα treatment, and provide evidences for IFIT1's potential in predicting efficiency of IFNα therapy.

Innovations and breakthroughs

The study clarifies the role of IFIT1 in the regulation of IFNα treatment for CHB by an epidemiologic study among Chinese patients. It highlights IFIT1's potential in predicting IFNα therapy's end point virological response.

Applications

The study identifies that polymorphism rs303218 on IFIT1 could be a predictor for the end point virological response of IFNα therapy, which may provide insight to the individualized treatment of CHB in the future.

Terminology

IFIT1: Interferon-induced protein with tetratricopeptide repeats 1. Virological response: Defined as serum HBV DNA level < 2000 IU/L at the end of the treatment.

Peer-review

The IFN induced proteins with tetratricopeptide repeats 1 is related gene which can be strongly induced by IFN type 1. It suppress cellular translation and was shown to block viral replication thus the importance to focus on such single nucleotide polymorphisms. The article represents an accepted population survey in an under-analysed population and contributes to the literature

important information for genetic, global association studies. Its impact is significant.

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ORIGINAL ARTICLE

Retrospective Study

Therapeutic efficacy and stent patency of transhepatic portal vein stenting after surgery

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Author contributions: Kim CW designed the study; Jeon UB analyzed the data and wrote the paper; Kim TU performed the data analysis; Choo KS, Jang JY and Nam KJ collected the data; Chu CW and Ryu JH designed the study, and revised the paper; all authors have read and approved the final version to be published.

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Abstract

AIM

To evaluate portal vein (PV) stenosis and stent patency after hepatobiliary and pancreatic surgery, using abdominal computed tomography (CT).

METHODS

Percutaneous portal venous stenting was attempted in 22 patients with significant PV stenosis (> 50%) - after hepatobiliary or pancreatic surgery - diagnosed by abdominal CT. Stents were placed in various stenotic lesions after percutaneous transhepatic portography. Pressure gradient across the stenotic segment was measured in 14 patients. Stents were placed when the pressure gradient across the stenotic segment was > 5 mmHg or PV stenosis was > 50%, as observed on transhepatic portography. Patients underwent follow-up abdominal CT and technical and clinical success, complications, and stent patency were evaluated.

RESULTS
Stent placement was successful in 21 patients (technical success rate: 95.5%). Stents were positioned through the main PV and superior mesenteric vein (*n* = 13), main PV (*n* = 2), right and main PV (*n* = 1), left and main PV (*n* = 4), or main PV and splenic vein (*n* = 1). Patients showed no complications after stent placement. The time between procedure and final follow-up CT was 41-761 d (mean: 374.5 d). Twenty stents remained patent during the entire follow-up. Stent obstruction - caused by invasion of the PV stent by a recurrent tumor - was observed in 1 patient in a follow-up CT performed after 155 d after the procedure. The cumulative stent patency rate was 95.7%. Small in-stent low-density areas were found in 11 (55%) patients; however, during successive follow-up CT, the extent of these areas had decreased.

CONCLUSION
Percutaneous transhepatic stent placement can be safe and effective in cases of PV stenosis after hepatobiliary and pancreatic surgery. Stents show excellent patency in follow-up abdominal CT, despite development of small in-stent low-density areas.

Key words: Liver; Vein; Stent; Computed tomography; Surveillance; Efficace

Core tip: Portal vein (PV) stenosis can occur after hepatobiliary and pancreatic surgery. Portal hypertension due to PV stenosis induces clinical manifestations in many organs, including intractable ascites, esophageal and gastrointestinal bleeding, and liver dysfunction. Percutaneous transhepatic stent placement can be safe and effective in cases of PV stenosis since stents show excellent patency on follow-up abdominal computed tomography despite the presence of small in-stent low-density areas.

Jeon UB, Kim CW, Kim TU, Choo KS, Jang JY, Nam KJ, Chu CW, Ryu JH. Therapeutic efficacy and stent patency of transhepatic portal vein stenting after surgery. *World J Gastroenterol* 2016; 22(44): 9822-9828 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9822.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9822

INTRODUCTION

Acquired portal vein (PV) stenosis is most commonly observed after liver transplantation (LT)[1-3]. In the non-transplant population, PV stenosis can present with pancreatitis[4] or tumor encasement[5], or as a postsurgical complication[6]. Portal hypertension from PV stenosis induces clinical manifestations in many organs, including intractable ascites, esophageal and gastrointestinal bleeding, and liver dysfunction[7].

Treatment options for patients with PV stenosis or occlusions are limited. In the past, PV complications were managed surgically, such as by thrombectomy or surgical revision. However, surgical management of these complications has been limited by technical difficulties due to the development of postsurgical fibrosis and the length of the involved venous structures[8]. Percutaneous interventional procedures have gained worldwide acceptance for alleviating the symptoms of portal hypertension, due to their minimal invasiveness and high success rates with low number of complications[9]. Abdominal computed tomography (CT) and Doppler ultrasound tests have been the main modalities of patient follow-up after this procedure; the reported stent patency rate has been relatively good, ranging between 60 and 100% during the first year[5,7,10,11]. Abdominal CT scans allow the visualization not only of the status of the original lesion, but also of changes in the PV stent. This study aimed to evaluate the therapeutic efficacy and stent patency of PV stenting after hepatobiliary and pancreatic surgery, using abdominal CT.

MATERIALS AND METHODS

Patients

From January 2011 to December 2012 at a single institution, percutaneous transhepatic stent placement was attempted in 22 patients [11 women, 11 men; mean age: 65.6 years (range: 26-78 years)] with significant PV stenosis (> 50% of the main PV diameter) diagnosed using abdominal CT after hepatobiliary or pancreatic surgery [pylorus-preserving pancreatoduodenectomy (*n* = 7), Whipple procedure (*n* = 6), distal pancreatectomy (*n* = 2), hepatectomy or hepatic segmentectomy (*n* = 6), and deceased donor liver transplant (*n* = 1)] (Table 1).

The reasons for surgery included pancreatic cancer (*n* = 7), common bile duct cancer (*n* = 3), Klatskin tumor (*n* = 2), colon cancer with duodenal invasion (*n* = 1), colon cancer with liver metastasis (*n* = 1), hepatocellular carcinoma (*n* = 1), gall bladder cancer (*n* = 1), gastrointestinal stromal tumor (*n* = 1), ampulla of Vater cancer (*n* = 1), chronic pancreatitis (*n* = 1), intrahepatic bile duct stone (*n* = 1), and hepatic failure after toxic hepatitis (*n* = 1). Clinical manifestations in patients with PV stenosis included intestinal angina-like abdominal pain (disabling mid-epigastric or central abdominal pain within 10-15 min after eating) and anorexia refractory to medical treatment, ascites, increased Jackson-Pratt (JP) drain output, and abnormal liver function test (LFT). The interval between surgery and stent placement was 5-274 d (mean: 34 d) (Table 2).

One patient was diagnosed with asymptomatic left PV stenosis after right hepatectomy, when the pressure gradient on transhepatic portography was 5 mmHg. She had undergone balloon angioplasty only and was excluded from this study.

Table 1 Types of surgery and lesion locations of the patients

patient No.	Age/sex	Surgery	Locations
1	65/F	DDLT	Main PV
2	74/F	Whipple's op	PV-SMV
3	58/M	Distal pancreatectomy + Spl-SMV bypass	PV-SMV
4	73/M	Whipple's op	PV-SMV
5	61/F	Whipple's op	PV-SMV
6	63/F	PPPD	PV-SMV
7	75/F	PPPD	PV-SMV
8	68/F	PPPD	PV-SMV
9	65/F	PPPD	PV-SMV
10	70/M	PPPD	PV-SMV
11	55/M	PPPD	PV-SMV
12	70/F	PPPD	Main PV
13	69/F	Whipple's op	PV-SMV
14	54/M	Right anterior segmentectomy	Right PV
15	76/M	Right hemicolectomy + PPPD	PV-SpV
16	26/M	Right lobectomy	Left PV
17	55/F	Right + caudate lobectomy	Left PV
18	66/M	Right + caudate lobectomy	Left PV
19	74/M	Subtotal pancreatectomy, Splenectomy, partial nephrectomy	PV-SMV
20	79/F	Trisegmentectomy + caudate lobectomy	Left PV
21	68/M	Whipple's op	PV-SMV
22	78/M	Central segmentectomy	Right PV

DDLT: Deceased donor liver transplant; PPPD: Pylorus-preserving pancreatoduodenectomy; PV: Portal vein; SMV: Superior mesenteric vein; SpV: Splenic vein.

Table 2 Clinical manifestations of patients

Clinical manifestations	No. of patients
Intestinal angina-like abdominal pain refractory to medical treatment	
Only pain	2
Worsening of PVS during the follow-up (> 2 wk)	4
Worsening of PVS during the follow-up (> 2 wk) + abnormal LFT	1
PVT after PCD	1
Abnormal LFT	1
Fail to PV anastomosis during the operation	1
Anorexia refractory to medical treatment (with increased JPDO)	1
Ascites	4
Increased JPDO	6
Abnormal LFT	1

"Worsening of PVS" indicates aggravation of the PVS on abdominal CT during the follow-up period. "Increased JPDO" indicates increased serosanguinous JP drain output–suspected ascites. CT: Computed tomography; JPDO: Jackson-Pratt drain output; PVS: Portal vein stenosis; PCD: Percutaneous drainage; PVT: Portal vein thrombosis.

Procedure

Informed consent was obtained from each patient. This retrospective study was approved by our institutional review board. All patients received local anesthesia consisting on subcutaneous injection of lidocaine and an intravenous injection of pentanyl (pentanyl citrate, Myung Moon Pharmaceutical; Seoul, Korea). Percutaneous transhepatic puncture of the intrahepatic PV was performed with a 21-gauge needle (PTC US needle, A&A M.D.; Seongnam, South Korea) under sonographic and fluoroscopic guidance. The needle was exchanged for a 5-French coaxial dilator, and an 8-French sheath was placed over a 0.035-inch guidewire. A 0.035-inch angled hydrophilic guidewire (Radifocus, Terumo, Tokyo, Japan) and a 5-French cobra catheter were used to traverse the PV stenosis. Direct main portography was performed with 12 mL of contrast medium injected into the patient's splenic or superior mesenteric vein (SMV) and the pressure gradient across the stenosis was measured. The criteria for PV stenting were as follows: stenosis > 50% of the main PV diameter revealed by transhepatic portography, or a pressure gradient across the stenosis > 5 mmHg (Table 3).

Table 3 The criteria for portal vein stenting - stenosis > 50% of the main portal vein diameter revealed by transhepatic portography, or a pressure gradient across the stenosis > 5 mmHg

Criteria	No. of patients
PVS > 50% with measuring of PrG	
PrG > 5 mmHg	11
PrG = 5 mmHg	
Contrast stagnation with collaterals formations	2
Kinking of PV (stenosis and acute angulation)	1
PVS > 50% without measuring of PrG	
Contrast stagnation with collaterals formations	3
Kinking of PV	3
Stenosis and partial PVT	1

PVS: Portal vein stenosis; PrG: Pressure gradient; PVT: Portal vein thrombosis.

Stent placement was performed using stents from the following vendors: Smart (Cordis; Miami Lakes, FL, United States), Zilver (Cook; Bloomington, IN, United States), Protégé (Covidien; St. Paul, MN, United States), Hercules (S&G Biotech Inc.; Seongnam, South Korea), Complete SE (Medtronic Inc.; Minneapolis, MN, United States), and Express LD (Boston Scientific; Natick, MA, United States). Stents of the same diameter or with a diameter 1-2 mm larger than that of the non-stenotic extrahepatic PV were used.

Balloon angioplasty following stent placement was not routinely performed; however, balloon angioplasty was performed in cases in which the deployed stent showed an hourglass deformity of < 50% its normal diameter or the pressure gradient across the stenosis was > 5 mmHg. Angioplasty was carefully performed using a balloon catheter of the same (or smaller than the deployed stent to prevent PV rupture.

After transhepatic portography and stent placement, the PV pressure gradient was measured to evaluate whether the stenosis or occlusion was resolved. The transhepatic parenchymal track was embolized with several 0.035-inch stainless steel coils (Cook; Bloomington, IN, United States), gelfoam slurry (Cutanplast, Mascia Brunelli; Milano, Italy), or n-butyl cyanoacrylate (NBCA; Histoacryl, B. Braun;

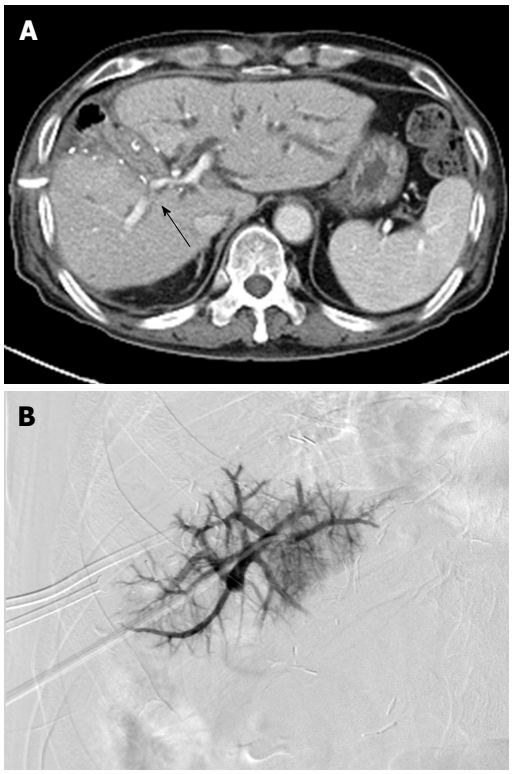

Figure 1 A selection failure case. A 78-year-old man (patient No. 23) presenting with right PV stenosis after hepatic central segmentectomy with liver metastasis from colon cancer. A: Axial CT scan 40 d after surgery showing severe stenosis (arrow) at the right PV. B: Percutaneous transhepatic portogram showing peripheral right PV. Main PV was not selected due to severe stenosis at the right PV. CT: Computed tomography; PV: Portal vein.

Melsungen, Germany) to prevent bleeding. No routine anticoagulation therapy was administered after the procedure.

Study endpoints

The following parameters were retrospectively documented: technical success, clinical success, development of complications, PV flow after stent placement, and pressure gradient across the area before and after stent placement. Technical success was defined as successful stent placement in the intended location in the PV with subsequent improvement in portal venous flow and < 30% residual stenosis. Clinical success was defined as improvement on patient clinical manifestations, such as nausea, abdominal pain, abnormal liver function, and ascites. Major complications included those requiring increased level of care or additional surgical or interventional manipulation and those resulting in adverse sequelae or death. Minor complications defined self-limiting events.

Patency of the PV stents was evaluated with follow-up abdominal CT 3-6 mo after the procedure and, after that, annually. According to clinician discretion, however, the follow-up schedule could be altered. Axial CT image of portal venous phase (5 mm-thick slice) was routinely used for evaluation. In cases in which the luminal changes caused by the stents were not fully visualized with axial images, some of the axial images were transferred to a workstation in which a PC-based three-dimensional reconstruction software (Aquarius iNtuition 4.4, TeraRecon; San Mateo, CA, United States) was installed. Three-dimensional image reconstruction was performed, which included volume-rendering and multiplanar or curved planar reformation along or across the venous segments of interest. Small in-stent low-density areas, such as the ones observed after carotid stenting[12], were observed between the stent wall and a contrast-filled PV on follow-up CT scans. Extent, location, and relative time of discovery were recorded.

Statistical analysis

Portal venous pressure gradient was analyzed before and after stent placement using the Wilcoxon rank test; cumulative stent patency rates were calculated using the Kaplan-Meier estimation. Statistical analysis was conducted using the SPSS software (version 18.0); a $P < 0.05$ indicates a statistically significant difference.

RESULTS

Stenting was successful in 21 out of 22 patients (technical success rate: 95.5%). Guidewire selection failed in 1 patient due to severe PV stenosis (Figure 1). All patients in whom PV stenting was successful (n = 21) showed improvement of clinical manifestations (*e.g.*, disappearance of intestinal angina, ascites, and abnormal LFT, and decreased JP drain output). Remarkably, 6 patients showed increased JP drain output during the early follow-up periods after surgery, with JP drain output decreasing rapidly within 1 wk after stenting. Therefore, the clinical success rate was of 100%. The used stents were 37-80 mm long and 8-14 mm in diameter. In 1 patient, 2 stents were used due to stent misplacement. Twenty-one self-expandable and 1 balloon-expandable stents were used, including the Smart (Cordis, Cordis, Inc.; Miami Lakes, FL, United States; n = 8), Zilver (Cook; Bloomington, IN, United States; n = 7), Protégé (Covidien; St. Paul, MN, United States; n = 3), Hercules (S&G Biotech Inc.; Seongnam, South Korea, n = 2), Complete SE (Medtronic Inc.; Minneapolis, MN, United States, n = 1), and Express LD (Boston Scientific; Natick, MA, United States; n = 1).

Stents were positioned as follows: through main PV and SMV (n = 13), through main PV (n = 2), through right PV and main PV (n = 1), through left PV and main PV (n = 4), and through main PV and splenic vein (n = 1).

The transhepatic parenchymal track was embolized with coils (n = 15), gelfoam slurry (n = 6) or n-butyl cyanoacrylate (n = 1) to prevent bleeding. There were no procedure-related complications, such as perihepatic hematoma, hemobilia, hemoperitoneum,

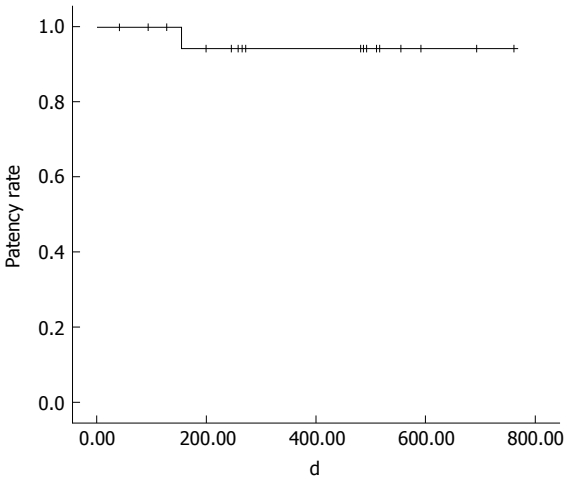

Figure 2 Kaplan-Meier results for cumulative patency rates among 22 patients who underwent portal vein stenting. Initial decrease in patency rate occurred within 155 d after procedure. Further decrease in patency rate was not observed during the entire follow-up period. The cumulative patency rate for PV stent was 95.7%. PV: Portal vein.

or pneumoperitoneum.

Pre- and post-stenting pressure gradients across the stenosis (measured in 14 patients) were 8.7 ± 3.4 mmHg (range: 5-16 mmHg) and 2.1 ± 1.1 mmHg (range: 1-5 mmHg), respectively. The decrease in pressure gradient after the procedure was statistically significantly (Wilcoxon rank test; $P < 0.05$).

The first follow-up CT examination was performed 1-162 d (mean: 40.1 d) after the procedure; sequential follow-up CT were performed in all patients. The interval between procedure and final follow-up CT was 41-761 d (mean: 374.5 d). Twenty stents remained patent during the entire follow-up period. One stent obstruction was identified on a follow-up CT scan 155 d after the procedure; this obstruction was caused by invasion of the PV stent by a recurrent tumor. In a study population, 8/17 patients with malignant tumors showed local recurrence of tumors, but 7 stents that were not directly invaded by the tumor remained patent during the entire follow-up period. The cumulative stent patency rate was 95.7% (Kaplan-Meier estimation; Figure 2). Follow-up CT performed 83-409 d (mean: 180.6 ± 99.5 d) after the procedure revealed small in-stent low-density areas in 11 patients (55%). In all cases, these areas were visualized in patients in whom the stents were placed through PV and SMV, within 25% of the luminal diameter. During consecutive follow-up CT, the extent of these areas slightly decreased (Figure 3). These low-density areas were located in the proximal and distal edge ($n = 9$) or mid portion ($n = 2$) of stents.

DISCUSSION

Postoperative PV stenosis is a surgical complication usually observed after concurrent resection and anastomosis of the PV after hepatobiliary and pancreatic surgery[7,13], and LT[1,9]. Percutaneous intervention through transhepatic approach was firstly introduced in LT patients by Olcott *et al*[14]. This technique has gained worldwide acceptance for the treatment of PV stenosis after LT due to their minimal invasiveness, low number of complications, and high success rate[15,16]. Nonetheless, the reported stenosis recurrence rate following balloon angioplasty alone has been relatively high (28.6%-36.8%). Stents have usually been used to treat recurrent and elastic portal venous stenoses following balloon angioplasty, as this procedure presents several potential complications, such as PV rupture or recoil[15,17,18]. Therefore, Ko *et al*[11] suggested performing primary stent placement rather than balloon angioplasty in the early post-transplantation period (< 1 mo) to minimize the potential need of repeat surgery and the risk of anastomotic rupture during balloon angioplasty. In our study, primary stenting was performed in all patients in the same way as done in the study by Ko *et al*[11], given that the interval between operation and stenting was relatively short. PV stent placement success in non-transplant populations was similar to that in the LT population[7,19,20].

In our study, all patients showed minor symptoms and signs of portal hypertension, such as nausea, intestinal angina-like pain, abnormal LFT, ascites, and increased JP drain output (no variceal bleeding was recorded). PV stenosis symptoms were identified after relatively short postoperative follow-up periods (within 2 mo, with the exception of 2 patients). Conservative management and follow-up was performed in patients presenting with intestinal angina only ($n = 5$, Table 1); however, PV stenosis in these cases was not improved during the follow-up period. Six patients presenting JP drains showed serosanguineous drainage output increase; these findings were different manifestations of ascites. Considering these observations, patients with significant PV stenosis presenting only minor symptoms, such as intestinal angina, ascites, or increased JP drain output, were recommended to be treated early. Woodrum *at al*[20] reported on a patient that presented with intestinal angina symptoms, with a mesenteric venogram showing near occlusion of the splenic vein and SMV. After PV stent placement, the patient's symptoms were completely relieved, which is in accordance with the results of our study.

The long-term patency of PV stents has been previously investigated. Reported stent patency was of 60%-100% during 1-2 years follow-up periods[5,7,10,11]. Kim *et al*[7] performed a stent patency comparison between groups of patients presenting benign and malignant stenosis and observed that the mean patency period in the benign stenosis group was higher than that of the tumor recurrence group. The shorter patency period in the tumor recurrence group seemed to be caused by a shorter survival period rather than by tumor ingrowth into the stent due to tumor recurrence. In our study, even in those cases in which local tumor recurrence was observed, the stent remained patent unless direct tumor invasion to the stent occurred; however, further follow-up was needed

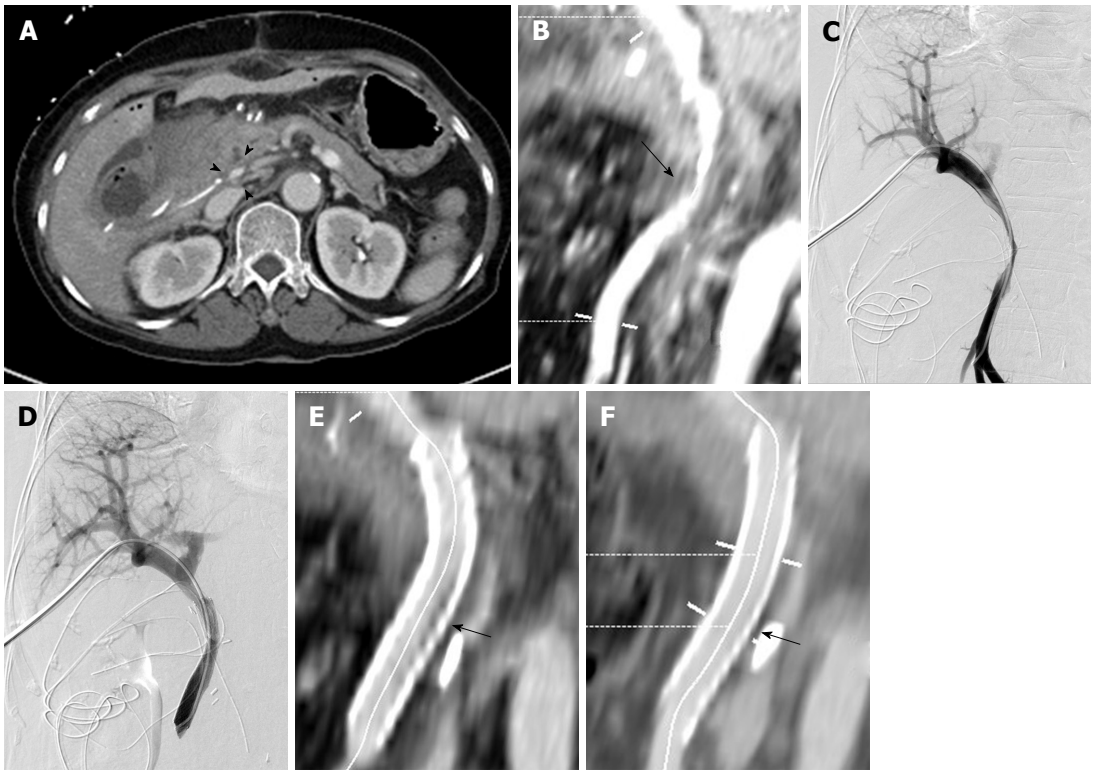

Figure 3 A 68-year-old woman (patient No. 8) presenting with main portal vein stenosis after pylorus-preserving pancreatoduodenectomy for duodenal gastrointestinal stromal tumor. She presented with anorexia and increased JP drain output during the follow-up period. A: Axial CT image 13 d after surgery showing stenosis at junction between the PV and SMV (arrowheads); B: Curved planar reformatted (CPR) image from abdominal CT 13 d after surgery showing stenosis at junction between the PV and SMV (arrow); C: Percutaneous transhepatic portogram showing severe stenosis (> 50%) at the PV-SMV junction. The pressure gradient was not measured because of definite stagnation of the contrast medium. The contrast medium injected at the distal SMV is clearly stagnant; D: Portogram showing metallic stent in the main PV and SMV and the elimination of stenosis. After the procedure, the patient's symptoms and signs improved; E: CPR image from abdominal CT 2 d after stenting showing patent stent with small in-stent low-density area (arrow); F: The extent of small in-stent low-density area decreased on CPR image from abdominal CT scan 555 d after stenting (arrow). CT: Computed tomography; PV: Portal vein; SMV: Superior mesenteric vein.

for these patients.

A pressure gradient > 5 mmHg across a stenosis has been considered "significant" in some reports[3,18,21]. However, no standard definition regarding a significant pressure gradient currently exists. In our study, the pressure gradient in 3 patients was < 6 mmHg. We observed aggravation of stenosis in one of these patients, kinking of PV in another, and diffuse stenosis (> 50%) in the third one. Several researchers have also reported clinically successful cases of stent placement following balloon angioplasty for the treatment of PV stenosis, using a pressure gradient < 6 mmHg[2,15,22]. Therefore, we consider that treatment is beneficial in patients who have symptoms related to PV inflow abnormalities or portal hypertension, even when the change in pressure gradient is not significant.

In some cases, small in-stent low-density areas were identified on follow-up CT scans, such as after carotid stenting[12]. The size of these areas decreased during consecutive follow-up CT. The exact pathology of these lesions was not fully understood but did not alter the patency of the stents. Additional studies are needed to clarify these observations.

The main limitations of this study are its retrospective nature and the small number of patients. Another limiting factor is that all procedures were usually performed based on clinical manifestations observed on CT and PV pressure gradient, not on clinical manifestations, such as intestinal angina and ascites; however, risk reduction of PV hypertension should also be considered in cases in which early intervention was recommended.

In conclusion, percutaneous transhepatic stent placement can be safe and effective in the treatment of PV stenosis developed after hepatobiliary and pancreatic surgery. Importantly, stents show excellent patency in follow-up abdominal CT scans, despite the development of small in-stent low-density areas.

COMMENTS

Background

Acquired portal vein (PV) stenosis is sometimes observed after hepatobiliary and pancreatic surgery, but treatment options for patients with PV stenosis or occlusions are limited. Surgical management of these complications has been limited by technical difficulties due to the development of postsurgical fibrosis and the lengths of the involved venous structures.

Research frontiers

Percutaneous interventional procedures have gained worldwide acceptance for their ability to alleviate the symptoms of portal hypertension because of their minimal invasiveness and high success rates, with low number of complications.

Innovations and breakthrough

This study aimed to evaluate the therapeutic efficacy and patency of PV stenting after hepatobiliary and pancreatic surgery, using abdominal CT.

Applications

Percutaneous transhepatic stent placement can be safe and effective for the treatment of PV stenosis after hepatobiliary and pancreatic surgery.

Terminology

Pressure gradient: pressure changes in two different vessels (*e.g.*, the PV and superior mesenteric vein). This can be checked between anatomical stenoses.

Peer-review

The authors report their experience with PV/SMV stenting in patients who had previous HPB surgical resections. The study is well written and interesting as there is very little in the medical literature on this topic.

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ORIGINAL ARTICLE

Observational Study

Attempt to calculate the prevalence and features of chronic hepatitis C infection in Tuscany using administrative data

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Author contributions: Silvestri C drafted the initial manuscript, participated in study design, in interpretation of the data, revised the article critically for important intellectual content; Bartolacci S, Pepe P and Monnini M participated in the acquisition and analysis of the data; Voller F and Cipriani F participated in study design and they were the guarantor of the methods used, revised the article critically for important intellectual content; Stasi C participated in study design, wrote the paper, interpreted the data, revised the article critically for important intellectual content; all authors have reviewed and approved the final manuscript.

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Data sharing statement: No additional data are available.

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Abstract

AIM

To evaluate this prevalence in Tuscan populations that was known and unknown to the Tuscan Regional Health Service in 2015.

METHODS

Tuscan Health administrative data were used to evaluate hepatitis C virus (HCV) infected people known to the Regional Health Service. Residents in Tuscany with a HCV exemption code (070.54) were identified. Using the universal code attributed to each resident, these patients were matched with hospital admission codes identified by the International Classification of Diseases, Ninth Revision (ICD-9), Clinical Modification, and with codes for dispensing drugs to patients by local and hospital pharmacies. Individuals were considered only once. Capture-recapture analysis was used to evaluate the HCV-infected population unknown to the Regional Health Service.

RESULTS

In total, 14526 individuals were living on 31/12/2015 with an exemption code for HCV. In total, 9524 patients were treated with pegylated interferon + ribavirin and/

or direct-acting antiviral drugs during the last 10 years, and 13879 total hospital admissions were noted in the last 15 years. After data linkage, the total number was 25918. After applying the Capture-Recapture analysis, the number of unknown HCV-infected people was 23497. Therefore, the total number of chronic HCV-infected people was 38643, excluding those achieved sustained virological response to previous treatment.

CONCLUSION

Our results show a prevalence of HCV infected people of 1%. Tuscan administrative data could be useful for calculating health care costs and health planning in the coming years.

Key words: Hepatitis C; Public health; Fibrosis; Antiviral treatment; Epidemiology

Core tip: Given the considerable differences among the world regions, the calculation of hepatitis C virus (HCV) prevalence through administrative flows seems to be essential for intervention policy strategies. Currently, these data are highly interesting given that the introduction of direct-acting antiviral drugs has highlighted the problem of sustainability due to the high costs of new drugs in low and middle income countries. Therefore, given the high number of chronic HCV-infected patients and the high costs of these drugs, the administrative data could be useful for calculating health care costs and health planning in the coming years.

Silvestri C, Bartolacci S, Pepe P, Monnini M, Voller F, Cipriani F, Stasi C. Attempt to calculate the prevalence and features of chronic hepatitis C infection in Tuscany using administrative data. *World J Gastroenterol* 2016; 22(44): 9829-9835 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9829.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9829

INTRODUCTION

The screening of blood donors, which began in the early 1990s, has reduced the spread of hepatitis C virus (HCV) in the population. The most effective preventative measures for HCV include the screening and testing of blood and organ donors, the implementation of practices in healthcare settings and a strong education programme. Bruggmann *et al*[1] in a recent systematic review, show that in selected countries (Australia, Austria, Belgium, Brazil, Canada, Czech Republic, Denmark, Egypt, England, France, Germany, Portugal, Spain, Sweden, Switzerland, Turkey) the viremic prevalence varied widely between countries, ranging from 0.3% in Austria, England and Germany to 8.5% in Egypt. A subsequent systematic review[2], considering 15 countries (Argentina, Finland, Greece, India, Ireland, Israel, Luxemburg, Mexico, Mongolia, The Netherlands, New Zeland, Norway, Poland, Russia, South Africa), shows that a viremic prevalence ranged from 0.13% in the Netherlands to 2.91% in Russia. Gower *et al*[3] revealed a 1.6% (range 1.3%-2.1%) global prevalence of people who have been infected with HCV (presence of anti-HCV antibodies), and this value reaches 2.0% if we exclusively consider the population over 15 years of age.

In most cases, the test for anti-HCV antibodies remains positive even in people who have cleared the virus, while a minimum percentage of these may instead be falsely positive.

On the contrary, the true prevalence is defined as the presence of HCV-RNA in serum or plasma. In this case, the estimated global HCV prevalence is 1.1% (range 0.9%-1.4%), whereas the value is 1.4% if we exclusively consider people over 15 years of age.

The meta-analysis of Gower *et al*[3] reveals very different prevalences of anti-HCV and HCV-RNA in different regions of the world, highlighting the strong variability in different areas (the lowest prevalence of 0.1% was observed in Oceania, whereas the highest prevalence of 4.1% was observed in Sub-Saharan West Africa). The World Health Organization (WHO) estimates that 150 million people, approximately 3% of the world population, are HCV chronically infected and are at an increased risk of developing liver cirrhosis and hepatocellular carcinoma[4].

The WHO estimates that 15 million people are currently infected with HCV in European countries[5].

Ansaldi *et al*[6] observed a slightly different sero-prevalence in Italy. The authors used the national serological bank of the "European Sero-Epidemiology Network - ESEN" project testing samples for anti-HCV, HCV-RNA and genotypes from 18 Italian regions. The analysis, which was performed by age and geographic area, showed that 52.6% of anti-HCV-positive subjects were also HCV-RNA positive, with a north-south gradient ranging from 1.3% to 4.6% and a greater involvement in the population over 30 years of age. The analysis revealed an overall anti-HCV prevalence of 2.7%.

One of the main features of HCV is its genetic variability. Seven genotypes are currently known and divided into subtypes. In Italy, four genotypes are the most common. As observed in the most of Europe, the most frequent is the 1b genotype, which has a prevalence of 60%, followed by 2c (20%), 1a (6%) and 4a/d (8%)[6]. Based on this premises, the aims of this study were to assess the prevalence and features of HCV infected patients through both data reported in research studies and administrative data and to calculate the number of patients eligible for treatment according to some of the Italian Medicines Agency prioritization's criteria.

MATERIALS AND METHODS

Calculation of prevalence

A first analysis was performed by applying the prevalence data reported by Ansaldi *et al*[6] on HCV-RNA-positive residents in Italy. National Statistical Institute (ISTAT) data were used for this calculation. A subsequent analysis was performed using prevalence data of Ansaldi *et al*[6] on the resident population in Tuscany (data of Regional Health Agency of Tuscany). Using the universal identification code (IDUNI) attributed by the Region Tuscany to every citizen residing in its territory, we identified people who were assigned a code (in each information flow - transmission of information from one "place" to another).

Therefore, the calculation of people with HCV infection known to the Regional Health System was performed using the main current sanitary flows of the Regional Health Agency of Tuscany. Through the universal code attributed by Region Tuscany to each person, these patients were matched with hospital admission codes identified by the International Classification of Diseases, Ninth Revision (ICD-9) Clinical Modification (such as acute hepatitis: ICD9-CM: 070.41 e 070.51; chronic hepatitis: 070.44 and 070.54; hepatocellular carcinoma: 155.0; cirrhosis: 571.5) and codes for dispensing drugs to patients by local and hospital pharmacies (ribavirin J05AB04, daclatasvir J05AX14, sofosbuvir J05AX15, simeprevir J05AE14, dasabuvir J05AX16, viekirax J05AX67, pegintron L03AB10, pegasys L03AB11, sofusbuvir and ledipasvir J05AX65). Each individual was considered only once. In the most part of cases in Tuscany the detection of anti-HCV was performed using the 3rd generation of anti HCV tests, while for the detection of HCV-RNA was used the real time PCR.

Calculation of comorbidity score

Charlson Comorbidity Index was applied to calculate the comorbidity score of these patients. This method includes 19 categories of comorbidity and predicts ten-year mortality for those patients who may have a range of co-morbid conditions. A score of 1, 2, 3 or 6 is assigned to each co-morbid condition, depending on the risk of dying associated with the condition. For a physician, this information is helpful to know how aggressively a condition must be treated. Highest scores indicate greatest comorbidities. For example, a score of > 3 is associated with a survival probability of 45% in 10 years[7].

Sustained virological responders

A number of patients treated with pegylated interferon (PEGINF) + ribavirin (RBV) achieved sustained virological response (SVR); therefore, the overall prevalence was calculated excluding such patients from our cohort.

For the computation of the prevalence without sustained virological responders, the different responses to drug treatment according to viral genotype were taken into account using percentages of sustained virological responders observed by Marcellin *et al*[8].

According to the authors, previous treatment led to a SVR 24 wk after the end of treatment in 41.8% of patients with genotype 1, 71.4% of those with genotype 2, and 60.6% of those with genotypes 3 and 4.

Therefore, based on the genotypic subdivision in Italy of patients treated with PEGINF + RBV, the prevalence of HCV patients was calculated excluding those patients who benefited from the treatment.

Patients unknown to the regional health service

To estimate the number of people with HCV who are unknown to the Regional Health System, Capture-Recapture analysis was used. This method is most useful to count all of the individuals in the population. A major application of this method is epidemiology, where the method is used to estimate the completeness of disease registers. In the basic formulation (in the case of two capture sources) the sample of HCV patients from a target population is captured, marked and released, and a second sample captured at some later time. Therefore, the number of HCV patients observed in each sample is noted, and it is possible to estimate the size of the total population. Log-linear model allows to extend this method to more than two sources and to treat dependence among the sources. It is one of the most used method to estimate the total population size. Backwards stepwise regression was used to find a model which adequately described the data with the least number of parameters and the final model was chosen according to Akaike Information Criteria (AIC). The final model include all the two-way interaction terms. The data were analysed using STATA12.

Calculation of patients eligible for treatment

In Italy, the Italian Drug Agency (AIFA) has identified a number of criteria for the prescription of second-generation DAAs to patients with HCV-related chronic disease. Among these, the 4 criteria provide the treatment of patients with chronic hepatitis with fibrosis stage METAVIR $\geqslant$ F3 (or corresponding ISHAK or corresponding liver stiffness $\geqslant$ 10 kPa).

Therefore, a liver stiffness $\geqslant$ 10 kPa can be a useful indicator to estimate how many people could be treated with DAAs along this criteria. For the Tuscan cohort, we used the study of Arena *et al*[9] and unpublished data of the Regional Health Agency of Tuscany. Out of 150 patients, 94 had a fibrosis stage $\leqslant$ F3, and 56 patients had a fibrosis stage $\geqslant$ F3.

RESULTS

Calculation of HCV prevalence

Tables 1 and 2 summarize the results obtained using

Table 1 Prevalence of anti-hepatitis C virus and hepatitis C virus-RNA by age in Italy and Tuscany on 01.01.2015

	Italy				Tuscany	
	Anti-HCV (%)	HCV-RNA^{+1} (%)	No. of resident people anti-HCV^{+}	No. of HCV-RNA^{+}	No. of resident people anti-HCV^{+}	No. of HCV-RNA^{+}
Geographic area						
North	1.3 (0.8-1.8)	39.1	361397	140945	108827	64317
Center	2.9 (1.7-4.1)	59.1	350628	206871		
South	4.6 (3.4-5.8)	56.0	961638	538517		
Age						
0-23 mo	3.8 (1.0-6.6)	14.3	58677[2]	8391[2]	3396[2]	486[2]
2-14 yr	0.8 (0.3-1.3)	66.6	54712[3]	36438[3]	3137[3]	2090[3]
15-30 yr	1.8 (1.0-2.6)	44.4	178552	79277	9696	4305
31-45 yr	5.9 (3.8-8.0)	48.3	767082	370500	46649	22532
46-60 yr	6.8 (3.9-9.7)	65.0	911233	592301	56568	36769
> 60 yr	5.2 (1.9-8.5)	66.6	836736	557266	73919	49230
Total	2.7 (2.2-3.2)	52.6	1641482	863419	108827	64317

[1]HCV-RNA positive % calculated on anti-HCV; [2]Using Istat data, the age range is 0-24 mo; [3]Using Istat data, the age range is 3-14 year. The total is not the sum of Cases. Source: Regional Health Agency of Tuscany on Ansaldi *et al*[6] prevalence and National Institute of Statistic data.

Table 2 Hepatitis C virus genotypes prevalence estimation in Italy and Tuscany

Genotypes	HCV-RNA	
	Italy (n = 863419)	Tuscany (n = 64317)
1b	518051	38590
2c	172684	12863
1a	51805	3859
3a	51805	3859
4a/d	69074	5145

Source: Regional Health Agency of Tuscany on Ansaldi *et al*[6] prevalence and National Institute of Statistic data.

Table 3 Hepatitis C virus - RNA resident population in Tuscany known to the Regional Health System by gender and age group n (%)

Age (yr)	Males	Females	Total
0-14	70 (0.5)	58 (0.5)	128 (0.5)
15-34	633 (4.3)	522 (4.6)	1155 (4.5)
35-44	1862 (12.8)	1104 (9.7)	2966 (11.4)
45-54	5270 (36.1)	2414 (21.3)	7684 (29.6)
55-64	2914 (20.0)	1743 (15.4)	4657 (18.0)
65-74	1920 (13.2)	2325 (20.5)	4245 (16.4)
75+	1918 (13.1)	3165 (27.9)	5083 (19.6)
Total	14587 (100)	11331 (100)	25918 (100)
	56%	44%	

Source: Regional Health Agency of Tuscany.

the prevalence of Ansaldi *et al*[6] in Italian and Tuscan people living as of 01/01/2015. (1) in total, 14526 people with an exemption code for HCV were living as of 31/12/2015; (2) In total, 9524 patients were treated with PEGINF + RBV and/or DAAs during the last 10 years; (3) the total number of hospital admissions in the last 15 years was 13879; and (4) after data linkage, the total number was 25918.

These people were already known to the Regional Health Service and considered as HCV-RNA positive. The characterization by gender and age revealed an increased prevalence in males compared with females (males: 56%; females: 44%), and the highest involvement was noted in individuals over 45 years of age (83.6%) (Table 3).

Table 4 Charlson Index score adjusted for age calculated on the estimated resident population in hepatitis C virus - positive patients from Tuscany known to the Regional Health System based on age group

Age (yr)	CHARLSON INDEX			
	0	1-2	3-4	⩾ 5
0-14	82	45	1	0
15-34	753	373	21	8
35-44	1.790	1.001	79	96
45-54	4.165	2.633	311	575
55-64	2.365	1.566	346	380
65-74	1.947	1.656	397	245
75+	1.850	1.974	885	374
Total	12.952	9.248	2.040	1.678
	50%	36%	8%	6%

Source: Regional Health Agency of Tuscany.

Calculation of comorbidity score

Applying the Charlson Comorbidity[7] Index to our cohort, 14% of known patients had a score ⩾ 5 (Table 4).

Sustained virological responders

Applying the prevalence of SVR observed by Marcellin *et al*[8] on the Tuscan population, 9524 individuals with chronic HCV infection treated with either PEGINF + RBV or DAAs were living as of 31/12/2015. Of these, the number of patients who benefited from the therapy and therefore did not need further treatment was 5652 (Table 5).

Patients unknown to the regional health service

Applying the Capture-Recapture analysis, it was estimated that 23497 people with chronic HCV infection living in Tuscany were unknown to the Regional Health Service. Therefore, by adding the 25918 people

Table 5 Patients known to the Regional Health Agency of Tuscany treated with pegylated interferon + ribavirin and/or direct-acting antiviral drugs

Genotypes	HCV patients treated with PEGINF + Rib	Sustained virological responders to PEGINF + Rib	Elegible patients to DAAs	HCV patients treated with DAAs	Sustained virological responders[1] to DAAs
1b (60%)	4516	2075	2445		
1a (6%)	452				
2c (20%)	1505	1075	364	1996	1896
3a (6%)	452	271	151		(about 95% SVR)
4a/d (8%)	603	247	300		
Totale	7528	3668	3260		

[1]HCV is considered regardless of genotypes. Sustained virological responders and total number of patients based on genotype - Source: Regional Health Agency of Tuscany and prevalence data by Marcellin *et al*[8]. PEGINF: Pegylated Interferon; RBV: Ribavirin; DAAs: Direct-acting antiviral drugs.

with chronic HCV infection known to the Regional Health Service and the 23497 people unknown to the Regional Health Service, a total of 49415 people with chronic HCV infection were identified in Tuscany.

Calculation of patients eligible to treatment

According to literature data, approximately 40% of Tuscan patients with chronic HCV have a liver stiffness > 10 kPa. Therefore, considering this value as a parameter for eligibility to DAAs along the 4 criteria, it is possible to assume that out of the 20266 chronic HCV-infected patients known to the Regional Health Service (25918 people with HCV infection known to Regional Health Service - 5652 who had SVR to previous treatment with PEGINF + RBV and DAAs), 8106 are potentially eligible for the 4 criteria that provide the treatment of HCV patients with fibrosis stage METAVIR ⩾ F3.

The same calculation was applied to patients to unknown to the Regional Health Service. Therefore, the total number of unknown patients to Regional Health Service was 18377.

Therefore, out of 38643 patients who were unknown and known to the Regional Health Service, 15457 are eligible for DAAs.

DISCUSSION

In 2010, a WHO resolution recognised viral hepatitis as a global health problem and stressed the need to implement measures for its prevention, diagnosis and treatment[10]. In May 24, 2014, a follow-up re-solution urged WHO Member States to develop and implement a national strategy based on epidemiological data[11]. Given the considerable differences among Italian regions, the calculation of HCV prevalence through administrative flows seems to be essential for intervention policy strategies. Currently, these data are highly interesting given the introduction of DAAs, which allow the eradication of the virus. In addition, these data have highlighted the problem of sustainability due to the high costs of new drugs in low and middle income countries. Intervention strategies are directed both towards the eradication of cases known and unknown to Regional Health Services, especially in the population at risk of contracting infection. There are various statistical methods to calculate all the individuals in the population that include both subjects who are known and subjects who are unknown to Regional Health Services. In this study, we used the log-linear model that allows correction for any covariates (such as sex and age). The total prevalence calculated for the prevalence observed in previous studies and that of administrative data are 1.7 *vs* 1.3 %. Currently, based on the calculation of patients with SVR to previous treatment with PEGINF + RBV and DAAs, a fraction of patients (10772) out of these 49,415 were sustained virological responders; therefore, the prevalence was 1%.

The raccomandations of the European Association for the Study of the Liver[12] outline that the goal of treatment is to cure HCV infected patients in order to prevent hepatic necro-inflammation, fibrosis, cirrhosis, decompensation of cirrhosis, HCC, severe extra-hepatic manifestations and death. However, because not every HCV-infected patient can be treated within the next year or so, the EASL suggest prioritization of the treatment based on fibrosis stage, risk of progression towards more advanced disease, presence of HCV-associated extra-hepatic manifestations and risk of HCV transmission (active injection drug users, men who have sex with men with high-risk sexual practices, women of childbearing age who wish to get pregnant, haemodialysis patients, and inmates). Along some of these reccomandations, treatment should be prioritized in patients with advanced fibrosis (METAVIR score F3 to F4), including patients with decompensated cirrhosis who have a contra-indication to the use of IFN-a but can be safely treated with IFN-free regimens.

The Italian Drug Agency published the prioritization's criteria for the therapy restricting DAAs treatment to groups of patients[13]. Some of these criteria include the stage of fibrosis, which is one of the most important predictors of progression of the disease that affects treatment decisions.

Currently, treatment is reimbursed for patients with post-liver transplantation (LT) recurrent HCV and

advanced graft fibrosis; patients with chronic HCV and cryoglobulinaemic syndrome or non-Hodgkin lymphoma; patients with chronic HCV and advanced fibrosis (METAVIR ⩾ F3, corresponding to liver stiffness ⩾ 10 kPa); patients with decompensated cirrhosis listed for LT with MELD < 25 or with HCC within Milan criteria and an expected time on the list of at least 2 mo; and patients with any (non-liver) organ transplantation and chronic HCV, with METAVIR ⩾ 2; and patients with METAVIR F0-F2 (only for Simeprevir)[13]. When we consider the eligibility criteria, 34,164 patients were eligible for DAAs based on the criteria, including HCV infected patients with advanced fibrosis (METAVIR ⩾ F3, corresponding to liver stiffness ⩾ 10 kPa). A large number of studies have suggested that liver stiffness measurement by transient elastography is a useful technique for diagnosing severe fibrosis and cirrhosis and for excluding significant fibrosis in hepatitis C virus patients[14,15].

In the entire cohort of patients Charlson Comorbidity score of > 3, which was associated with a survival probability of 45% over 10 years, was observed in 14% of people.

For drug prescription, clinicians also considered comorbidities to exclude an interaction between DAAs and other drugs prescribed for comorbidities. However, interferon- and ribavirin-treatment free regimens are available and require shorter treatment periods (3 mo). These regimens do not have substantial side effects and are also effective in patients with decompensated cirrhosis[16].

Recently, Wedemeyer *et al*[17] and Gane *et al*[18] used a mathematical model to forecast HCV disease burden. The results of these analyses suggest that the largest reduction in HCV-related morbidity and mortality occurs when increased treatment is combined with higher efficacy therapies, generally in combination with increased diagnosis. However, for most countries, this will require a 3-5 fold increase in diagnosis and/or treatment. Using today's treatment paradigm, HCV-related mortality and morbidity is expected to increase in all countries with the exception of France, which has had a high treatment rate[17]. The analysis of Wedemeyer *et al*[17] and Gane *et al*[18] also demonstrated that with a treatment rate of approximately 10%, it is possible to achieve elimination of HCV (> 90% decline in total infections by 2030). However, treatment of F0-F1 patients was necessary if the goal of the strategy is to eliminate HCV.

In conclusion, Tuscan administrative data reveal a prevalence of chronic HCV infection of approximately 1%. Given the high number of chronic HCV-infected patients and the high costs of these drugs, the new regimens will probably remain inaccessible to many patients throughout the world. Therefore, these data could be useful for calculating health care costs and health planning in the coming years.

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ORIGINAL ARTICLE

Observational Study

Clinical features of HBsAg seroclearance in hepatitis B virus carriers in South Korea: A retrospective longitudinal study

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Abstract

AIM

To investigate the characteristic features of hepatitis B surface antigen (HBsAg) seroclearance among Korean hepatitis B virus (HBV) carriers.

METHODS

Carriers with HBsAg seroclearance were selected by analyzing longitudinal data collected from 2003 to 2015. The period of time from enrollment to the negative conversion of HBsAg (HBsAg-NC) was compared by stratifying various factors, including age, sex, hepatitis B e antigen (HBeAg), HBV DNA, sequential changes in the signal-to-cutoff ratio of HBsAg (HBsAg-SCR), as measured by qualitative HBsAg assay, and chronic liver disease on ultrasonography (US-CLD). Quantification of HBV DNA and HBsAg (HBsAg-QNT) in the serum was performed by commercial assay.

RESULTS

Among the 1919 carriers, 90 (4.7%) exhibited HBsAg-NC at 6.2 ± 3.6 years after registration, with no differences observed among the different age groups. Among these carriers, the percentages of those with asymptomatic liver cirrhosis (LC) and hepatocellular carcinoma (HCC) at registration were 31% and 7.8%, respectively. The frequency of HBsAg-NC significantly differed according to the HBV DNA titer and US-CLD. HBeAg influenced HBsAg-NC in the 40-50 and 50-60

year age groups. HBsAg-SCR < 1000 was correlated with an HBsAg-QNT < 200 IU/mL. A gradual decrease in the HBsAg-SCR to < 1000 predicted HBsAg-NC. Six patients developed HCC after registration, including two before and four after HBsAg-NC. The rate at which the patients developed new HCC after HBsAg seroclearance was 4.8%. LC with excessive drinking and vertical infection were found to be risk factors for HCC in the HBsAg-NC group.

CONCLUSION

HCC surveillance should be continued after HBsAg seroclearance. An HBsAg-SCR < 1000 and its decrease in sequential testing are worth noting as predictive markers of HBsAg loss.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis B Surface antigen; HBsAg; Seroconversion; Hepatitis B e antigen; HBeAg; Liver cirrhosis

Core tip: In South Korea, where most hepatitis B virus carriers are infected with genotype C, hepatitis B surface antigen (HBsAg) seroclearance rate is 4.7%, and the incidence of hepatocellular carcinoma (HCC) after HBsAg loss is 4.8%. In patients with HBsAg seroclearance, the percentages of asymptomatic liver cirrhosis (LC) and HCC are 31% and 7.8% at enrollment, respectively. A signal-to-cutoff ratio of the qualitative HBsAg level of less than 1000 and its sequential decrease are worth noting as predictive markers of HBsAg loss. HCC surveillance should be continued after HBsAg seroclearance, particularly in patients with LC.

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INTRODUCTION

Hepatitis B virus (HBV) is the most important cause of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in endemic areas worldwide[1,2]. The natural course of HBV infection is associated with immunological changes that occur in three phases: tolerance, eradication, and recovery[3]. These phases are classified based on the serum aminotransferase level, hepatitis B e antigen (HBeAg) and HBV DNA titers, which represent hepatitis and viral replication, respectively[4,5]. Recovery is defined as ceasing of the self-replicating activity of the HBV genome and its transition to a non-replicating state. In general, a serum HBV DNA level of below 2000 IU/mL is considered to indicate an inactive hepatitis B surface antigen (HBsAg) carrier state[3,5-7]. Once the HBV genome is inactivated, it remains inert throughout life, HBeAg becomes negative, and HBsAg is cleared in approximately 40% of patients after 25 years of follow-up[3]. On the other hand, a significant proportion of carriers with HBeAg loss harbor the G1896A mutation, the so-called e-minus mutation[2]. In Korea, where most HBV carriers harbor genotype C2[8-10], most carriers over the age of 40 are infected with HBV with basal core promoter (BCP) double mutations (A1762G and A1764T), and more than half of these individuals have the G1896A mutation[9,10]. These mutations are associated with HBeAg-negative chronic hepatitis that is frequently reactivated[2,11,12], and HBeAg seroconversion is associated with the development of LC and HCC in two-thirds of carriers[2,7,13]. Because the turning point of seroconversion generally occurs near the age of 40[11], the recovery phase and timing of mutations usually overlap with the development of LC and HCC at this time[2,14]. However, HCC may also develop after HBsAg seroclearance[2,14]. These results highlight the difficulty of determining when and how the negative conversion of HBsAg (HBsAg-NC) in the serum takes the risk out of HCC. Thus, Korean HBV carriers represent a good model to study the clinical significance of HBsAg seroclearance in individuals with genotype C. This study investigated the long-term process of HBsAg seroclearance to elucidate the outcomes and predictive factors.

MATERIALS AND METHODS

Patients

Among chronic HBV carriers who visited the Hepatology Center of Bundang Jesaeng General Hospital between March 2003 and September 2015, all patients with HBsAg seroclearance were recruited. The clinical and laboratory data were retrospectively recorded at baseline and during follow-up. Chronic carriers were defined as those with HBsAg positivity on two successive tests performed at least six months apart. Registration or entry was defined as the initial visit to the center. The baseline data included the data collected at the first visit. HBsAg seroclearance was defined as two consecutive negative HBsAg tests performed at least six months apart.

Clinical data at baseline and during follow-up

The patients underwent tests for biochemical liver function, hepatitis B virological markers, and alpha-fetoprotein, as well as liver ultrasonography (US). The virological markers assessed included HBsAg, anti-HBs, HBeAg, anti-HBe, and HBV DNA. The anti-HBs or anti-HBe test was performed as clinically indicated. The anti-HCV test was performed at baseline and was repeated if the alanine aminotransferase level was elevated to more than twice the upper limit of normal.

Table 1 Rate and time interval of hepatitis B surface antigen seroclearance from entry

Age group	Total number (sex ratio)	[1]HBsAg seroclearance No. (rate/yr/sex ratio)	Time interval to HBsAg seroclearance (yr, mean ± SD)
< 30	181 (1.8:1)	4 (2.2%/0.55%/3.0:1)	3.99 ± 2.91
30-40	423 (2.3:1)	11 (2.6%/0.45%/1.2:1)	5.79 ± 4.23
40-50	574 (2.9:1)	36 (6.3%/0.92%/4.0:1)	6.87 ± 3.5
50-60	455 (0.4:1)	22 (4.8%/0.79%/2.7:1)	6.15 ± 3.89
> 59	286 (1.1:1)	17 (5.9%/1.05%/0.9:1)	5.64 ± 3.14
Total	1919 (1.4:1)	90 (4.7%/0.76%/1.9:1)	6.2 ± 3.6

[1]The overall rate of HBsAg seroclearance was 4.7%, and the estimated annual rate of HBsAg seroclearance was 0.76%. The rate of HBsAg seroclearance was significantly higher in the 40-50 and 50-60 year age groups than in the 20-30 and 30-40 year age groups (P = 0.001). Male predominance was observed in the 40s and 50s age groups but was not clear in the > 59 year age group. The time interval from entry to HBsAg loss was longer in the 40-50 and 50-60 year age groups than in the 20-30 and 30-40 year age groups.

The signal-to-cutoff ratio of the HBsAg (HBsAg-SCR) level was measured for each patient by qualitative assay.

Methods for testing virological markers

The HBsAg, anti-HBs, HBeAg and anti-HBe levels were determined by ARCHITECT qualitative assay, which is a chemiluminescent microparticle immunoassay (CMIA) that measures the resulting chemiluminescent reaction as relative light units (RLUs) (Abbott Laboratories, North Chicago, IL, United States). There is a direct relationship between the amount of HBsAg in a sample and the RLUs detected by ARCHITECT assay (Abbott Laboratories). The anti-HCV levels were determined using a third-generation enzyme immunoassay (Abbott Laboratories). HBV DNA was tested using a commercially available real-time polymerase chain reaction (RT-PCR; Roche Molecular Systems, Pleasanton, CA, United States), according to the manufacturer's instructions. The lower limit of detection for the Roche assays is 20 IU/mL. The viral load data obtained with the Roche assay were converted to international reference units for analysis using approximations for 1 pg (283000 copies) and 1 IU/mL (5.8 copies/mL).

Quantitative HBsAg assay

The data on the HBsAg levels in 177 patients were separately collected to perform correlation analysis with the HBsAg-SCRs obtained with CMIA. The HBsAg quantification (HBsAg-QNT) was measured by electrochemiluminescence immunoassay using an Elecsys HBsAg II Quant Assay (Roche Diagnostics, Indianapolis, IN, United States), according to the manufacturer's instructions. This approach quantifies HBsAg against an internal World Health Organization reference standard in IU/mL.

Statistical analysis

To compare the characteristics between groups, either the χ^2 test or Fisher's exact test was used to analyze categorical variables, and Student's *t*-test or Wilcoxon nonparametric test was used to analyze continuous variables. Statistical data were expressed as the mean ± SD. The correlation between the HBsAg-SCR and HBsAg-QNT was analyzed. The statistical procedures were performed with R-project version 3.2[15]. P values of < 0.05 were considered significant.

RESULTS

HBsAg seroclearance rate

Negative seroconversion of HBsAg was observed in 90 of 1919 carriers (4.7%), with an estimated annual rate was 0.76%. The rate of HBsAg loss was higher in the carriers over the age of 40 than in the younger carriers (5.7% *vs* 2.5%, P = 0.001). Among the carriers with HBsAg seroclearance, male predominance was more significant in the 40-50 and 50-60 year age groups than in the 30-40 and > 60 year age groups (P < 0.0001). The period of time until HBsAg clearance after registration was 6.48 ± 3.76 years. This value did not significantly differ among the age groups (P = 0.148) (Table 1).

Roles of HBeAg, the HBV DNA titer and BCP mutations in HBsAg seroclearance

Among the 90 carriers with HBsAg seroclearance, 82.2% were HBeAg-negative at registration. Among these HBeAg-negative carriers, 95.3% had no history of anti-viral treatment and were considered to be in the inactive phase. The time interval to HBsAg clearance did not differ between the HBeAg-positive and negative carriers (P = 0.982). Among the carriers with HBsAg seroclearance who had a treatment history, the time interval to HBsAg clearance did not differ between the HBeAg-positive and negative carriers (8.13 ± 3.12 *vs* 7.95 ± 3.45 years, P = 0.4442). However, this time interval was longer for the carriers who had received treatment than for those who had not received treatment (7.95 ± 3.34 *vs* 5.52 ± 3.48 years, P = 0.017), suggesting that anti-viral therapy does not lead to HBsAg seroclearance[16,17]. On the other hand, the time interval from entry to HBsAg seroclearance was significantly longer for the HBeAg-positive carriers than for the HBeAg-negative carriers in the 40s and 50s age groups (9.24 ± 2.49 years *vs*

Table 2 Comparison of the time interval to hepatitis B surface antigen seroclearance between carriers with and without hepatitis B e antigen at entry

Age group	HBeAg-positive		HBeAg-negative	
	n (%)	Years (mean ± SD)	*n* (%)	Years (mean ± SD)
< 30	1 (25)	8.15	3 (75)	2.6 ± 1.06
30-40	4 (36.4)	5.87 ± 4.9	7 (63.6)	5.75 ± 4.21
40-50	6 (16.7)	9.24 ± 2.84	30 (83.3)	6.4 ± 3.46
50-60	2 (9.1)	9.22 ± 1.71	20 (90.9)	5.84 ± 3.93
> 59	3 (17.6)	3.38 ± 1.81	14 (82.4)	6.12 ± 3.2
Total	16 (17.8)	7.23 ± 3.71	74 (82.2)	5.98 ± 3.2

Overall, the time interval of HBsAg seroclearance did not differ between the two groups (P = 0.982). However, when limited to the 40s and 50s age groups, the time interval to HBsAg seroclearance was significantly longer in the HBeAg-positive group than in the HBeAg-negative group (P = 0.013), but it did not significantly differ in the other age groups. In the 60s age group, the time interval to HBsAg seroclearance did not differ between the HBeAg-positive and negative groups (P = 0.1208).

6.18 ± 3.63 years, P = 0.013) (Table 2). Likewise, the time interval was significantly longer for the carriers with an HBV DNA level of > 2000 IU/mL than for those with a level of < 20 IU/mL (8.31 ± 3.26 years *vs* 4.92 ± 3.36 years; P = 0.0005) (Table 3). Data on the BCP double mutations, A1762G and A1764T, and the e-minus mutation, G1896A, were available for 18 patients. Women were predominant in the wild-type BCP group, whereas men were predominant in the mutant group (male/female = 1/6 *vs* 9/2, respectively; P = 0.0014). The BCP mutation carriers tended to be older than the wild-type carriers (41.9 ± 10.8 years *vs* 48.5 ± 7.6 years old, P = 0.0725). However, the time interval from entry to HBsAg seroclearance did not differ between the groups.

Utility of the signal-to-cutoff ratio of the qualitative HBsAg level

A log-linear-shaped correlation was detected between the HBsAg-SCR and HBsAg-QNT (Figure 1A), and a good correlation with a linear drift curve was observed between an HBsAg-SCR < 1000 and an HBsAg-QNT < 100 IU/mL (y = 0.0674x + 0.9902, R^2 = 0.916) (Figure 1B). No correlation was observed between these two values in the patients with HBsAg-QNT levels of over 200 IU/mL (Figure 1C). An inverse correlation was observed in the patients with very high HBsAg titers of over 10000 IU/mL (Figure 1D), indicating a prozone effect on the HBsAg-SCR caused by excess antigen. Based on the HBsAg-SCR value of 1000, we arbitrarily divided the 90 carriers with HBsAg seroclearance into two groups. The period of time from entry to HBsAg seroclearance was significantly shorter in the < 1000 group than in the ⩾ 1000 group (5.07 ± 3.74 years *vs* 7.47 ± 3.05 years, P = 0.0008). Moreover, a sequential decrease in the HBsAg-SCR was predictive of HBsAg-NC in every case (Figure 2). The HBeAg-positive rate was significantly higher in the ⩾ 1,000 group than in the < 1000 group (25% *vs* 11.9%, P = 0.0057). Further, the mean age in the < 1000 group tended to be higher than that in the ⩾ 1000 group (46.2 ± 11 years *vs* 50 ± 11.1 years old, P = 0.0593). However, there was no difference in the sex ratio or the prevalence of HCC or LC between the two groups.

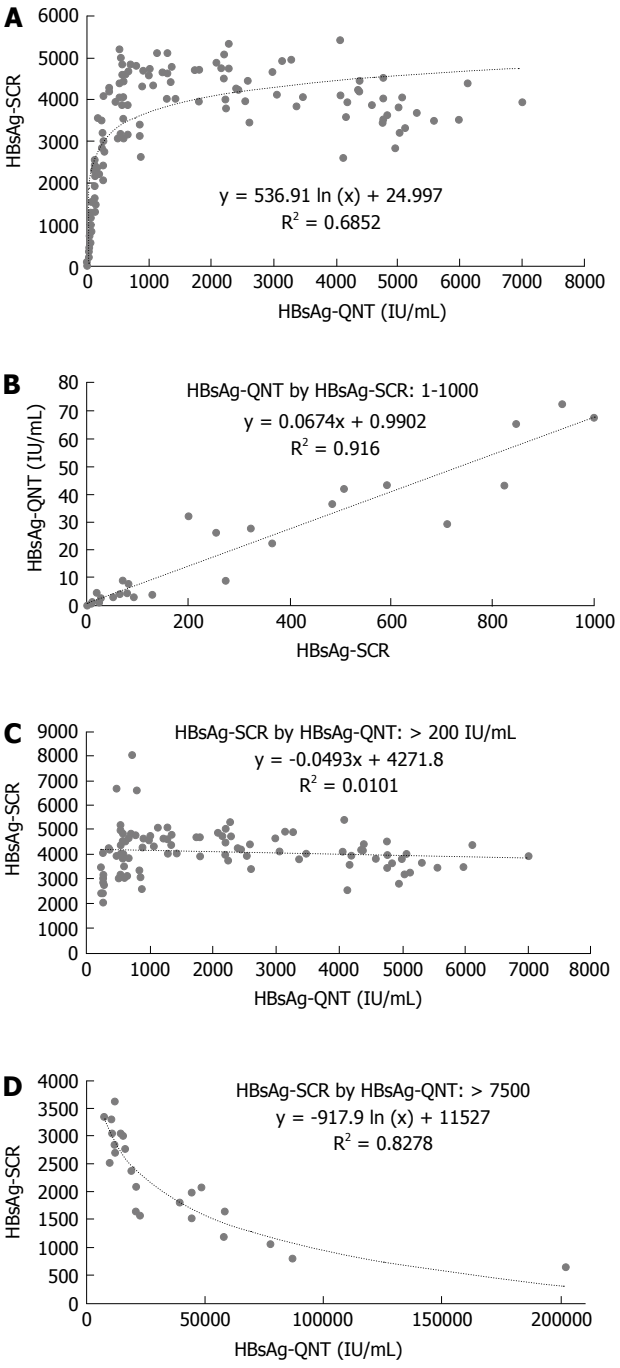

Figure 1 Signal-to-cutoff ratio of hepatitis B surface antigen was measured by qualitative assay and partially reflects the quantity of hepatitis B surface antigen in the serum. A: There was a log-linear correlation between the HBsAg-SCR and HBsAg-QNT; B: There was a good correlation with a linear drift curve between an HBsAg-SCR < 1000 and an HBsAg-QNT < 100 IU/mL (y = 0.0674x + 0.9902, R^2 = 0.916); C: HBsAg-QNT levels of greater than 200 IU/mL were not correlated with the HBsAg-SCR; D: Very high HBsAg titers of more than 10000 IU/mL were inversely correlated with the HBsAg-SCR, which was caused by a prozone effect. HBsAg-SCR: Signal-to-cutoff ratio of HBsAg; HBsAg-QNT: Quantity of HBsAg; HBsAg: Hepatitis B surface antigen.

Table 3 Comparison of the time interval to hepatitis B surface antigen seroclearance in each age group with the serum hepatitis B virus DNA titer at entry

	HBV DNA < 20[1] IU/mL		HBV DNA 20-2000 IU/mL		HBV DNA > 2000 IU/mL	
Age group	No.	Years (mean ± SD)	No.	Years (mean ± SD)	No.	Years (mean ± SD)
< 30	2	2.31 ± 1.32	2	5.67 ± 3.51	0	0
30-40	4	6.42 ± 4.82	4	3.72 ± 4.01	3	7.72 ± 3.92
40-50	12	5.73 ± 3.73	14	6.71 ± 3.36	10	8.47 ± 3.11
50-60	12	3.94 ± 2.99	6	8.36 ± 3.43	4	9.47 ± 3.11
> 59	9	5.07 ± 2.85	5	5.95 ± 3.28	3	6.82 ± 4.6
SUM	39	4.92 ± 3.36	31	6.45 ± 3.49	20	8.31 ± 3.26

[1]The 20 IU/mL value is the cut-off level for HBV DNA detected by real-time polymerase chain reaction. The time interval to HBsAg seroclearance was very significantly reduced in the HBV DNA < 20 IU/mL group compared with the > 2000 IU/mL group (P = 0.0005). The difference in the time interval to HBsAg seroclearance was slightly significant between the HBV DNA < 20 IU/mL and 20-2000 IU/mL groups and between the HBV DNA > 2000 IU/mL and 20-2000 IU/mL groups (P = 0.0671 and P = 0.0624, respectively).

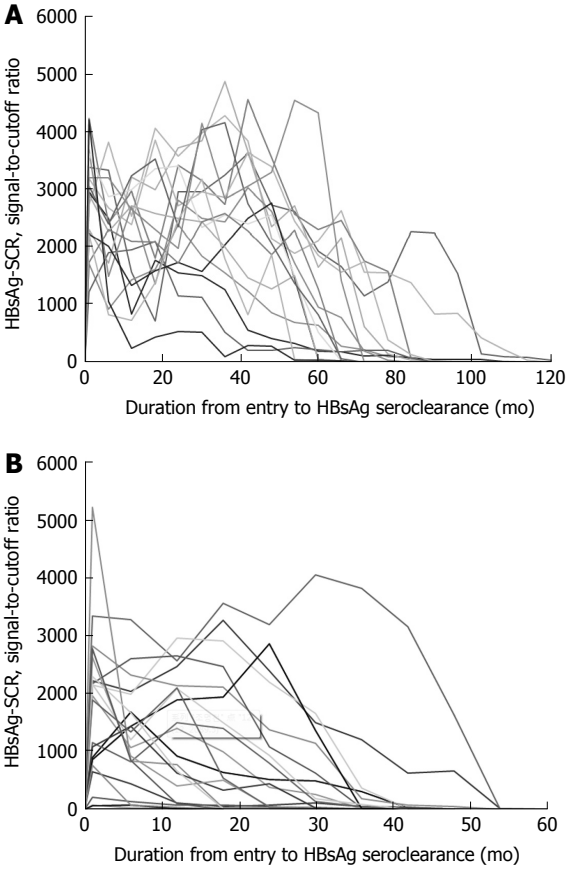

Figure 2 Sequential changes in the signal-to-cutoff ratio of Hepatitis B surface antigen in the patients with negative conversion of Hepatitis B surface antigen during the follow-up period after registration. A: HBsAg-SCRs in the patients with HBsAg-NC after 5 years post-entry; B: HBsAg-SCRs in the patients with HBsAg-NC before 5 years post-entry. HBsAg-SCR: Signal-to-cutoff ratio of HBsAg; HBsAg-NC: Negative conversion of HBsAg; HBsAg: Hepatitis B surface antigen.

LC and HBsAg seroclearance

We classified the patients with HBsAg seroclearance into the following three groups according to their US findings: normal, non-cirrhotic chronic liver disease (CLD) and cirrhotic. The period of time from study entry to HBsAg loss was significantly shorter in the normal US group than in the CLD and cirrhotic US groups (5.05 ± 3.92, 6.86 ± 3.51 years and 6.8 ± 2.98 years, respectively; normal US *vs* CLD, P = 0.0259; normal *vs* cirrhotic US, P = 0.0366) (Table 4). Among the 90 patients with HBsAg seroclearance, 31% had asymptomatic, inactive LC without HCC at registration. Men were predominant in the 30s (3/3) and 40s (9/10) age groups, but the proportion of females was increased in the 50s (3/5) and 60s (5/10) age groups. Five patients (17.9% of 28; male/female = 4/1) developed HCC during follow-up, including one before and four after HBsAg loss.

Hepatocellular carcinoma and HBsAg seroclearance

Among the 90 patients with HBsAg seroclearance, thirteen were diagnosed with HCC, including seven (7.8%) at registration (HCC group-1) and six (6.7%) during follow-up (HCC group-2). Among the patients in HCC group-2, two developed HCC prior to HBsAg loss, and four developed HCC at 0.66 ± 0.63 years after HBsAg loss, corresponding to a rate of 4.8% among the 83 carriers without HCC at entry. There was no difference in the time interval from entry to HBsAg loss between HCC group-1 and group-2 (6.78 ± 2.77 years and 5.77 ± 3.18 years, respectively). In addition to LC, the risk factors for HCC included excessive drinking in three men and vertical infection in one woman.

DISCUSSION

We summarize here five important key findings regarding HBsAg seroclearance in Korean HBV carriers from a new perspective. First, HBsAg-SCR, HBeAg, HBV DNA and CLD are factors associated with the time interval from a given carrier state to HBsAg seroclearance. Second, the start of a decrease in HBsAg-SCR usually indicates the gradual loss of HBsAg quantity. Third, asymptomatic, inactive LC is present in approximately 30% of carriers with HBsAg seroclearance. Fourth, HCC can develop after HBsAg loss. Fifth, in addition to LC, risk factors for HCC may include excessive drinking and a family history of HBV infection.

The HBsAg clearance rate seems to be lower in Korea than in other countries. The clearance rate was determined to be 0.76% in Korea, while other studies

Table 4 Comparison of the time to hepatitis B surface antigen seroclearance in each age group with the ultrasonography stage of chronic liver disease

Age group	Normal US		Non-cirrhotic CLD		Cirrhotic US	
	No.	Year (mean ± SD)	No.	Year (mean ± SD)	No.	Year (mean ± SD)
< 30	2	2.29 ± 1.28	2	5.7 ± 3.47	0	-
30-40	9	5.48 ± 4.59	1	5.56	1	5.6
40-50	9	5.7 ± 4.07	16	7.2 ± 3.63	11	7.35 ± 2.86
50-60	9	5 ± 4.23	8	6.59 ± 4.3	5	7.51 ± 2.38
> 59	3	3.81 ± 1.49	7	6.91 ± 3.17	7	5.14 ± 3.42
SUM	32	5.05 ± 3.92	34	6.86 ± 3.51	24	6.8 ± 2.98

Chronic liver disease (CLD) showing coarse parenchymal texture in ultrasonography (US). The time interval to HBsAg seroclearance was significantly shorter in the carriers with normal US than in those with non-cirrhotic CLD (US-CLD) or cirrhotic US (US-LC). There was no difference in the time interval to HBsAg seroclearance between the carriers with US-CLD and those with US-LC. Age did not significantly impact the difference in the time interval to the disappearance of HBsAg: normal US *vs* US-CLD, $P = 0.0259$; normal US *vs* US-LC, $P = 0.0366$; and US-CLD *vs* US-LC, $P = 0.4724$. However, there was no significant difference among the 40s and 50s age groups according to the stage of CLD: normal US *vs* US-CLD, $P = 0.091$; normal US *vs* US-LC, $P = 0.0678$; and US-CLD *vs* US-LC, $P = 0.3579$.

have reported rates of 1.15%-1.6% in Taiwan[6,18], 1.14% in Kawerau of New Zealand[19], 1%-1.9% in Caucasian carriers[5,17], 2.5% in the Goto Islands of Japan[20], and 3.08% in China[21]. Compared with data from Taiwan[18], HBsAg seroclearance in Korea was significantly reduced in all age groups as follows: 0.55% *vs* 0.77% in the 20-30 year, 0.45% *vs* 1.07% in the 30-40 year, 0.92% *vs* 1.65% in the 40-50 year, and 0.89% *vs* 1.83% in the 50 and over age groups. This variability may be due to various factors, such as geographic differences in genotypes, age, gender, viral loading and fibrosis at enrollment. For instance, almost all Korean carriers are known to be infected with genotype C, whereas 60% and 34% of Taiwanese carriers are infected with genotypes B and C, respectively[6,22].

Carriers with an initial HBeAg-positive result show the gradual negative conversion of HBeAg and HBV DNA before the loss of HBsAg[14]. In carriers with an initial HBeAg-negative result, HBV DNA is cleared from the serum before HBsAg-NC, although low HBV DNA titers are persistently detected in some patients[23]. These results are inter-related in that the HBsAg loss is usually preceded by a long period of inactive disease[17].

The time period from entry to HBsAg loss in our study is similar to that reported in a previous six-year study conducted in China[21]. Notably, no significant differences in this time interval were observed according to age, the HBeAg status or the HBV DNA titer at the time of enrollment. Two opposite conclusions have been made regarding the role of HBV DNA in HBsAg seroclearance. One is that HBV DNA is not a dependent factor for HBsAg-NC[6,19], while the other suggests that lower viral loads are predictive of HBsAg seroclearance[24]. In our study, the HBV DNA titer was associated with the relative period of time from detection of viral activity to HBsAg seroclearance, and this period was at least 1.5 times longer in the carriers with HBV DNA levels ⩾ 2000 IU/mL than in those with levels < 20 IU/mL 40s. However 50s, HBeAg exhibited this effect only in the 40s and 50s age groups, indicating its age-dependent role in predicting HBsAg loss, in contrast with the HBV DNA titer.

The baseline HBsAg level is known to be a better predictor of HBsAg seroclearance than other factors[6,16,19,21,24-26]. This statement applies to inactive carrier, in whom a lower HBsAg level is more predictive of HBsAg seroclearance. Reportedly, the optimum cut-off baseline HBsAg level has varied among studies, for example, studies have reported levels of < 10 IU/mL[6,21], < 100 IU/mL[19,24], < 200 IU/mL[25,26], and < 751 IU/mL[16]. In addition, it has been reported that the predictive capacity of the HBsAg level can be improved by considering it in combination with other factors, such as a normal platelet count[21], old age[19], an undetectable HBV DNA level[24], the HBV DNA level at 12 mo after HBeAg seroconversion[27], and a yearly ⩾ 0.5 log IU/mL reduction[25,26].

Because the quantitative HBsAg test is expensive, we analyzed the utility of the HBsAg-SCR in predicting HBsAg seroclearance. A good linear correlation was observed between an HBsAg-SCR < 1000 and HBsAg-QNT < 200 IU/mL. In addition, a prozone effect on the HBsAg-SCR caused by excess antigen was observed among the carriers with excessive HBsAg titers of > 10000 IU/mL, most of whom had a very high serum HBV DNA level of > 7 log10 IU/mL. With the exception of those carriers with highly replicative infections, the HBsAg-SCR was significantly reduced in the HBeAg-negative carriers compared with the HBeAg-positive carriers. Moreover, the period of time from a given viral state to HBsAg loss tended to be shorter, as shown in Figure 2. A gradual decrease in the repeated tests during follow-up usually indicated HBsAg loss within five years. These results suggest that sequential HBsAg-SCR data are very useful for predicting HBsAg seroclearance.

The rate of LC at entry of 31.1% for the carriers with HBsAg seroclearance is similar to that reported

previously[14]. These results indicate that LC is present in an appreciably high rate of carriers with HBsAg seroclearance. On the other hand, significant fibrosis has been reported to be more prevalent in patients with HBsAg seroclearance who are > 50 years of age compared with those who are < 50 years of age[19,28]. In our study, the prevalence of LC was significantly increased among the males who were approximately forty years of age. In addition, the carriers in whom LC/CLD was detected on US exhibited a longer time interval from entry to HBsAg seroclearance than those with normal US results. These results suggest that the correlation between age and HBsAg loss is meaningful when it is considered together with gender and LC. In addition, the frequency of HBsAg loss was higher in the carriers over the age of 40 than in the younger carriers, and this trend is similar to trends reported in Taiwan[18], Japan[20], and New Zealand[29]. These results might be associated with a high rate of HBsAg seroclearance during long-term follow-up, as reported in Chu and Liaw's study[18].

Inactive carriers generally have a good prognosis. Indeed, none of the patients developed HCC following HBsAg loss (median follow-up of 72 mo) in a community study conducted in Kawerau, New Zealand[19], or in a study of Caucasians[5]. In contrast, in studies performed in Hong Kong and the United States, 1.4%-2.4% of the patients developed HCC after HBsAg seroclearance[7,14,28]. In our study, 4.82% of the carriers with HBsAg seroclearance developed HCC after HBsAg loss, indicating that HBsAg seroclearance does not guarantee patient safety out of HCC.

In one study, HBsAg seroclearance in individuals < 50 years of age has been shown to be associated with a lower risk of the development of HCC[28]. In another study, a low baseline level of albumin and family histories of HBsAg positivity and HCC have been demonstrated to be associated with a high risk of development of HCC, even in individuals who are < 50 years of age at the time of HBsAg clearance[14]. In our study, HCC developed in one patient over 50 years of age and in three patients over 60 years of age after HBsAg seroclearance. All patients had asymptomatic LC at registration and no evidence of deterioration of liver function during follow-up. In addition, none of these patients had a family history of HCC. Three men were excessive drinkers, and one woman had a vertical HBV infection.

In conclusion, HBsAg seroclearance does not indicate safety out of HCC, particularly in patients with LC. Spontaneous HBsAg loss might occur in a large proportion of cryptogenic LC and HCC cases in Korea, and surveillance should be continued after HBsAg loss in the same manner as for HBsAg-positive patients. Sequential HBsAg-SCR data measured with the conventional test are very useful for the long-term management of carriers, similar to HBsAg levels.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection follows a unique natural course, consisting of immune tolerance, eradication, and a recovery phase. Intricate changes in the balance between host and viral factors stimulate the phase transition. Prognosis of chronic carriers is determined by a complicated process with consideration of phase transition. Seroclearance of hepatitis B surface antigen (HBsAg) is the final step of the recovery phase. However, the clinical features of HBsAg seroclearance have not yet been completely elucidated.

Research frontiers

The recovery phase usually overlaps with the occurrence of escape mutations in the HBV genome and the development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Thus, it is difficult to determine when and how the negative conversion of HBsAg (HBsAg-NC) has occurred to a sufficient extent to have preventive effects for HCC. The research hotspot is clarification of the clinical features associated with spontaneous HBsAg seroclearance, including the outcome and predictive markers, to facilitate the development of a patient management strategy both during and after the recovery phase.

Innovations and breakthroughs

The results of the present study have suggested that the phase transition to recovery occurs spontaneously in most patients and that asymptomatic LC is prevalent among these patients (31%). Thus, a significant proportion of chronic carriers are at risk of HCC at the time of recovery. In fact, the data showed that HCC developed among the patients with LC during the recovery phase both pre- and post-HBsAg seroclearance. On the other hand, it is known that the HBsAg quantity, in combination with the HBV DNA titer, is a predictive marker for HBsAg seroclearance. The authors have demonstrated for the first time that a signal-to-cutoff ratio of the qualitative HBsAg level (HBsAg-SCR) of < 1000 and its sequential decrease are notable predictive markers of HBsAg loss. Our data support the value of sequential HBsAg-SCR data.

Applications

The results of our study may facilitate the design of a management strategy for HBV carriers both pre- and post-HBsAg seroclearance. In particular, patients with evidence of chronic liver disease or LC should be carefully monitored for the development of HCC, even after the spontaneous loss of HBsAg.

Terminology

Serum HBsAg positivity lasting for six months is a serological indicator for chronic HBV infection. HBsAg seroclearance refers to the spontaneous HBsAg-NC in the serum. The HBsAg-SCR is the signal-to-cutoff ratio of HBsAg, as measured by qualitative assay, and the quantification of HBV DNA and HBsAg (HBsAg-QNT) is the quantity of HBsAg in the serum, as measured by quantitative assay. The HBsAg-SCR is determined according to the signal intensity of an antigen-antibody reaction. The authors found that carriers with a very high HBV DNA titer of > 7 logs IU/mL exhibited a prozone effect, in which the HBsAg-SCR was paradoxically reduced by excess antigen. In contrast, in the HBV DNA-negative patients, an HBsAg-SCR < 1000 was strongly correlated with an HBsAg-QNT < 200 IU/mL. Otherwise, the HBsAg-SCR was not correlated with the HBsAg-QNT.

Peer-review

This invited manuscript written by Park *et al* investigated the clinical features of HBsAg serological clearance among almost 2000 Korean HBV carriers. This is a valuable study that included large-scale clinical sample analyses; the significant conclusion of this study is that liver cancer surveillance should be continued after HBsAg seroclearance, particularly in patients with cirrhosis. The title describes the contents of the paper. The abstract is informative and completely self-explanatory; it briefly presents the topic, states the scope of the experiments, provides the significant data, and notes the major findings and conclusions. The purpose or purported significance of the article is explicitly stated. The research study methods are complete enough to enable the experiments to be reproduced. All figures and tables are necessary and appropriate. The discussion interprets the findings in view of the results

obtained in this and in past studies on this topic.

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ORIGINAL ARTICLE

Observational Study

Disease-specific miR-34a as diagnostic marker of non-alcoholic steatohepatitis in a Chinese population

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Author contributions: Pan Q, Zhang RN and Fan JG designed the research; Yan SY, Sun C, Xu ZJ and Chen YW collected material and clinical data from the patients; Liu XL, Zhang RN and Shen F performed the assays; Liu XL analyzed the data; Liu XL and Fan JG wrote the manuscript.

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Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Xin Hua Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

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Abstract

AIM

To assess disease-specific circulating microRNAs (miRNAs) in non-alcoholic steatohepatitis (NASH) patients.

METHODS

A total of 111 biopsy-proven non-alcoholic fatty liver disease (NAFLD) or chronic hepatitis B (CHB) patients and healthy controls from mainland China were enrolled to measure their serum levels of miR-122, -125b, -146b, -16, -21, -192, -27b and -34a. The correlations between serum miRNAs and histological features of NAFLD were determined. The diagnostic value of miRNA in NASH and significant fibrosis was analyzed and compared with that of cytokeratin-18 (CK-18), fibrosis-4 (FIB-4), and aspartate aminotransferase to platelet ratio index (APRI), respectively.

RESULTS

Circulating miR-122, -16, -192 and -34a showed differential expression levels between NAFLD and CHB patients, and miR-34a had an approximately 2-fold increase in NAFLD samples compared with that of CHB samples ($P < 0.01$). Serum miR-122, -192 and -34a

levels were correlated with steatosis (R = 0.302, 0.323 and 0.470, respectively, $P < 0.05$) and inflammatory activity (R = 0.445, 0.447 and 0.517, respectively, $P < 0.01$); only serum miR-16 levels were associated with fibrosis (R = 0.350, $P < 0.05$) in patients with NAFLD. The diagnostic value of miR-34a for NASH (area under the receiver operating characteristic, 0.811, 95%CI: 0.670-0.953) was superior to that of alanine aminotransferase, CK-18, FIB-4 and APRI in NAFLD, but miR-16 showed a limited performance in the diagnosis of significant fibrosis in NASH.

CONCLUSION

Circulating miR-34a may serve as a disease-specific noninvasive biomarker for the diagnosis of NASH.

Key words: MicroRNA; Non-alcoholic steatohepatitis; Hepatic fibrosis; Biomarker; Chronic hepatitis B

Core tip: Circulating miR-122, -192, -34a and -16 showed differential expression levels between non-alcoholic fatty liver disease and chronic hepatitis B patients, and serum miR-122, -192 and -34a could also differentiate non-alcoholic steatohepatitis (NASH) from non-alcoholic fatty liver. The latter were correlated with steatosis and inflammatory activity, and only serum miR-16 was associated with hepatic fibrosis. The diagnostic value of miR-34a for NASH was superior to that of alanine aminotransferase and cytokeratin-18, but miR-16 showed a slightly poorer performance than that of fibrosis-4 and aspartate aminotransferase to platelet ratio index in the diagnosis of significant fibrosis in NASH.

Liu XL, Pan Q, Zhang RN, Shen F, Yan SY, Sun C, Xu ZJ, Chen YW, Fan JG. Disease-specific miR-34a as diagnostic marker of non-alcoholic steatohepatitis in a Chinese population. *World J Gastroenterol* 2016; 22(44): 9844-9852 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9844.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9844

INTRODUCTION

With advances in the quality of life and various lifestyle transitions, non-alcoholic fatty liver disease (NAFLD) has become a growing public health concern worldwide, with a prevalence of about 15% in the general population of China[1]. NAFLD refers to a disease spectrum with progressive histological changes ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), which is characterized by hepatocyte injury with the presence of hepatic steatosis. If left untreated, NASH will result in irreversible liver damage, such as fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[2]. Currently, NASH is the most common cause of chronic liver disease and the second most common indication for liver transplantation in America[3]. Thus, there is an urgent need for improved detection of NASH. Invasive liver biopsy remains the gold standard for the diagnosis of NASH, and thus there is a strong interest in the development of non-invasive biomarkers for this disease, such as serum cytokeratin-18 (CK-18) and ferritin[4,5]. However, these serum biomarkers do not have sufficient sensitivity or specificity to act as robust predictors of NASH[6].

Chronic hepatitis B (CHB), a global health burden caused by hepatitis B virus (HBV) infection, affects approximately 350 million people worldwide, and 780000 die each year due to HBV-related diseases[7]. In Asia and the western Pacific, especially China, CHB is still a leading cause of cirrhosis and HCC[8-10]. Given the high prevalence of CHB in China, it cannot be excluded in the identification of biomarkers of NAFLD and NASH.

Recently, circulating microRNAs (miRNAs) have emerged as attractive candidate biomarkers for early detection and monitoring of liver disease progression and response to treatment, as they are protected from RNases in the body fluids and are extremely stable[11]. The use of miRNAs as noninvasive biomarkers of NAFLD is of particular interest. Recent studies have identified a series of miRNAs that are dysregulated in NAFLD, among which miR-122, -125b, -146b, -16, -21, -192, -27b and -34a are the most widely reported to contribute to the development of NAFLD and NASH[12-17].

Although many studies have been conducted to identify the biomarkers and mechanisms associated with NAFLD, many issues remain unsolved. For example, there is still a lack of reliable disease-specific biomarkers for the diagnosis of NASH. Therefore, we assessed whether circulating miRNAs can distinguish NAFLD from CHB and also diagnose NASH and significant fibrosis in NAFLD. In the present study, we analyzed the serum expression profile of eight miRNAs (miR-122, -125b, -146b, -16, -21, -192, -27b and -34a) in healthy controls, CHB controls and NAFLD patients from mainland China.

MATERIALS AND METHODS

Study design, patients and controls

This study was carried out in 111 Chinese Han individuals between May 2012 and May 2014. The inclusion criteria were as follows: (1) age of 18-60 years-old; (2) NAFLD group: evidence of hepatic steatosis by histology; and (3) CHB group: serum hepatitis B surface antigen (HBsAg) positivity for at least 6 mo, a liver biopsy showing chronic hepatitis with moderate or severe necro-inflammation, and patients not meeting the diagnostic criteria for cirrhosis or steatosis. The exclusion criteria were as follows: (1) excessive alcohol consumption (140 g per week for men, 70 g for women); (2) use of hepatotoxic medications or herbal

products; (3) other viral hepatitis; (4) diabetes; and (5) pregnancy or lactation. The healthy control group consisted of healthy age- and sex-matched subjects from our institutional staff with normal body mass index (BMI; calculated as body weight/height^2), liver enzymes, and abdominal ultrasonography findings. The Ethics Committees of Xinhua Hospital approved this study, and all the subjects provided informed written consent prior to enrollment.

Clinical and laboratory assessment

Clinical information, including age, sex, BMI and frequency and average daily consumption of alcohol, was collected at the time of liver biopsy. Blood samples were drawn from an antecubital vein after overnight fasting; the blood analysis, including platelet (PLT) count and levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL), were determined with an automatic biochemical analyzer. The fibrosis-4 (FIB-4) index was calculated as follows: age (y) × AST (U/L)/[platelet count (10^9/L) × ALT (U/L)]$^{1/2}$[18]. The AST to PLT ratio index (APRI) was calculated as AST (× upper limit of normal)/PLT (10^9/L) × 100[19]. HBsAg was detected by a commercially available enzyme immunoassay (Abbot Laboratories, Chicago, IL, United States). Additionally, the serum CK-18 M30 and M65 levels were measured using a human CK18-M30 enzyme linked immunosorbent assay (ELISA) Kit (U-1197; Peviva, Nacka, Sweden) and a CK18-M65 ELISA Kit (U-2098; Peviva), respectively.

Histopathological analysis

The liver biopsy specimens were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin (HE) and Masson. An experienced hepatopathologist, who was blinded to the clinical data, analyzed the liver biopsies. The steatosis score (S; 0-3) assessed the quantities of large or medium-sized lipid droplets (S0: < 5%; S1: 5%-33%; S2: 34%-66%; S3: > 67%). The activity grade (A; 0-4) was the unweighted addition of hepatocyte ballooning (0-2) and lobular inflammation (0-2). The fibrosis stage (F; 0-4) was assessed as follows: F0: none; F1: 1a or 1b perisinusoidal zone 3 or 1c portal fibrosis; F2: perisinusoidal and periportal fibrosis without bridging; F3: bridging fibrosis; and F4: cirrhosis. NASH was diagnosed based on the steatosis, activity, fibrosis (SAF) score of the fatty liver inhibition of progression (FLIP) algorithm ($S \geqslant {}_1A \geqslant {}_2F_{any}$)[20].

miRNA quantitation

Total miRNA was isolated from the serum using a miRNeasy Mini Kit (No. 217004; Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA was synthesized using a TaqMan MicroRNA Reverse Transcription Kit (ABI, Foster City, CA, United States). We performed quantitative real-time polymerase chain reaction (qRT-PCR) on a 7900 HT Sequence Detection System (ABI) using a *Taq*Man Universal PCR Master Mix (ABI) to assess the distribution of eight miRNAs in serum. The miRNA abundance of miR-122, -125b, -146b, -16, -21, -192, -27b and -34a was normalized to that of miR-1228 and was calculated based on the comparative $2^{\Delta\Delta Ct}$ method. The relative miRNA expression levels were transformed into their natural logarithm to eliminate heteroscedasticity.

Statistical analysis

All continuous variables are presented as the mean ± standard error of the mean. Statistical comparisons among groups were performed by a one-way analysis of variance followed by a Newman-Keuls post-test for continuous variables and by a χ^2 test for categorical variables. Correlations between serum miRNAs and hepatic histological features were assessed using Spearman's correlation. A P value < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curves were plotted, and the area under the ROC (AUROC) was calculated with a 95% confidence interval (CI) to identify the diagnostic efficacy. Calculations were performed using SPSS version 16.0 (Chicago, IL, United States), and graphs were generated using GraphPad Prism Software version 6.0 (San Diego, CA, United States). The statistical methods used in this study were reviewed by Dr. Guang-Yu Chen from the Clinical Epidemiology Center of Shanghai Jiao Tong University School of Medicine.

RESULTS

Characteristics of the study subjects

The characteristics of the study subjects are summarized in Table 1 and Table 2. Based on the SAF score, 31 patients were diagnosed with NASH. Age and sex were comparable among the groups. The NAFLD group displayed a significantly higher BMI compared with that of the control and CHB groups, and the NASH group had higher ALT and AST levels than those of the control and NAFL groups (P < 0.05). CK-18 (M30) and CK-18 (M65) levels were higher in the NASH group than in the NAFL group (P < 0.001), but there was no significant difference between the NAFLD and CHB groups (Figure 1).

Serum miRNA profiles in different groups

All eight miRNAs, except for miR-125 and -27b, were up-regulated in the NAFLD group compared with that of the controls (P < 0.05), with miR-34a being even higher and miR-122, -16 and -192 being lower in the NAFLD group compared with that of the CHB group (P < 0.01; Figure 2A). Among them, miR-122, -192 and -34a displayed the highest fold-change in NAFLD compared with the controls, which were 7.9, 4.0 and

Table 1 General and biochemical parameters of chronic hepatitis B and Non-alcoholic fatty liver disease patients

Parameters	Group		
	Control (n = 37)	CHB (n = 26)	NAFLD (n = 48)
General parameters			
Age (yr)	43.8 ± 1.3	40.1 ± 2.7	38.1 ± 1.8
Male n (%)	18 (48.6)	16 (61.5)	35 (72.9)
BMI (kg/m^2)	21.4 ± 0.3	23.0 ± 0.6[1]	26.9 ± 0.5[1,2]
Biochemical parameters			
ALT (U/L)	19.1 ± 2.4	138.2 ± 30.8[1]	68.7 ± 7.4[1,2]
GGT (U/L)	19.1 ± 1.9	83.8 ± 16.7	110.5 ± 35.9
TC (mmol/L)	4.5 ± 0.1	4.5 ± 0.2	4.9 ± 0.1
TG (mmol/L)	0.8 ± 0.1	1.7 ± 0.2[1]	2.1 ± 0.2[1]
HDL (mmol/L)	1.4 ± 0.0	1.1 ± 0.1[1]	1.2 ± 0.0[1]
LDL (mmol/L)	2.4 ± 0.1	2.5 ± 0.1	2.9 ± 0.1[1,2]

Data are expressed as mean ± SEM. [1]P < 0.05 *vs* the control group; [2]P < 0.05 *vs* the chronic hepatitis B (CHB) group. NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; GGT: γ-glutamyl transpeptidase; TC: Total cholesterol; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein.

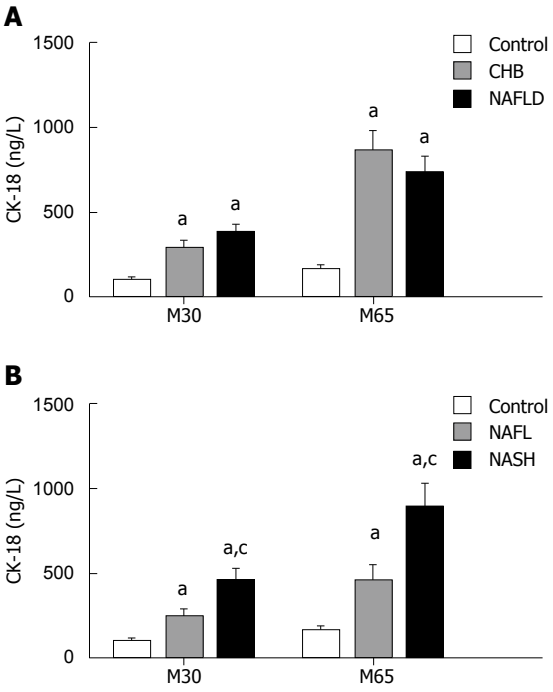


Figure 1 Serum cytokeratin-18 (M30) and cytokeratin-18 (M65) levels in chronic hepatitis B (A) and non-alcoholic fatty liver disease patients, as well as non-alcoholic fatty liver and non-alcoholic steatohepatitis (B) patients. CK-18 (M30) and CK-18 (M65) showed a higher level in non-alcoholic steatohepatitis (NASH) patients than non-alcoholic fatty liver (NAFL) patients (P < 0.001), but they showed no significant difference between the non-alcoholic fatty liver disease (NAFLD) patients and chronic hepatitis B (CHB) patients. [a]P < 0.05 *vs* the control group; A: [c]P < 0.05 *vs* the CHB group; B: [c]P < 0.05 *vs* the NAFL group. CK-18: Cytokeratin-18.

2.8, respectively. When we divided NAFLD patients into NAFL and NASH subgroups, the results showed that miR-122, -192 and -34a had significant differences not only between the NAFL and the control groups but also between the NASH and the NAFL groups (P < 0.001; Figure 2B).

Table 2 General and biochemical parameters of non-alcoholic fatty liver and non-alcoholic steatohepatitis patients

Parameters	Group		
	Control (n = 37)	NAFL (n = 17)	NASH (n = 31)
General parameters			
Age (yr)	43.8 ± 1.3	37.1 ± 3.1	38.1 ± 2.3
Male n (%)	18 (48.6)	13 (76.4)	22 (71.0)
BMI (kg/m^2)	21.4 ± 0.3	26.3 ± 0.9[1]	27.2 ± 0.6[1]
Biochemical parameters			
ALT (U/L)	19.1 ± 2.4	47.8 ± 6.2[1]	80.2 ± 10.4[1,2]
AST (U/L)	18.8 ± 1.4	30.2 ± 3.8	51.5 ± 6.0[1,2]
GGT (U/L)	19.1 ± 1.9	78.2 ± 36.8	128.2 ± 51.9
PLT (× 10^9/L)	200.4 ± 11.3	237.0 ± 14.8	207.3 ± 9.1
TC (mmol/L)	4.5 ± 0.1	4.7 ± 0.2	5.0 ± 0.1
TG (mmol/L)	0.8 ± 0.1	1.9 ± 0.3[1]	2.2 ± 0.3[1]
HDL (mmol/L)	1.4 ± 0.0	1.2 ± 0.1[1]	1.2 ± 0.1[1]
LDL (mmol/L)	2.4 ± 0.1	2.9 ± 0.2[1]	2.9 ± 0.1[1]

Data are expressed as mean ± SEM. [1]P < 0.05 *vs* the control group; [2]P < 0.05 *vs* the non-alcoholic fatty liver (NAFL) group. NASH: Non-alcoholic steatohepatitis; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyl transpeptidase; PLT: Platelets; TC: Total cholesterol; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein.

Correlations between miRNAs and hepatic histological features

A correlation analysis was performed between serum miRNAs and hepatic histological features in NAFLD patients (Table 3). miR-122, -192 and -34a were correlated with hepatic steatosis (R = 0.302, 0.323 and 0.470, respectively, P < 0.05) and inflammatory activity (R = 0.445, 0.447 and 0.517, respectively, P < 0.01). Notably, miR-34a showed the strongest correlation with lobular inflammation (R = 0.552, P < 0.01), and miR-122 showed the strongest association with hepatocellular ballooning (R = 0.477, P < 0.01). However, only miR-16 was associated with hepatic fibrosis (R = 0.350, P < 0.05; Figure 3).

Predictive value of miRNAs in NASH and significant fibrosis

We estimated the value of the eight miRNAs in the diagnosis of NASH and significant fibrosis in patients with NAFLD using a logistic regression model. Upon adjustment for BMI, miR-34a was shown to be a significant predictor of NASH (P < 0.01) and miR-16 was found to be a significant predictor of significant fibrosis (≥ F2; P < 0.05). However, none of the eight miRNAs could predict advanced fibrosis (≥ F3) or cirrhosis. The diagnostic performance of miR-34a for NASH and miR-16 for significant fibrosis was evaluated using ROC analysis. Interestingly, the AUROC of miR-34a for NAFLD was very high (0.811, 95%CI: 0.670-0.953; Figure 4A). Although serum ALT, M30 and M65 had a specificity of 0.937 for the diagnosis of NASH, their sensitivities, which were 0.481, 0.407 and 0.519, respectively, were relatively poor. Fortunately,

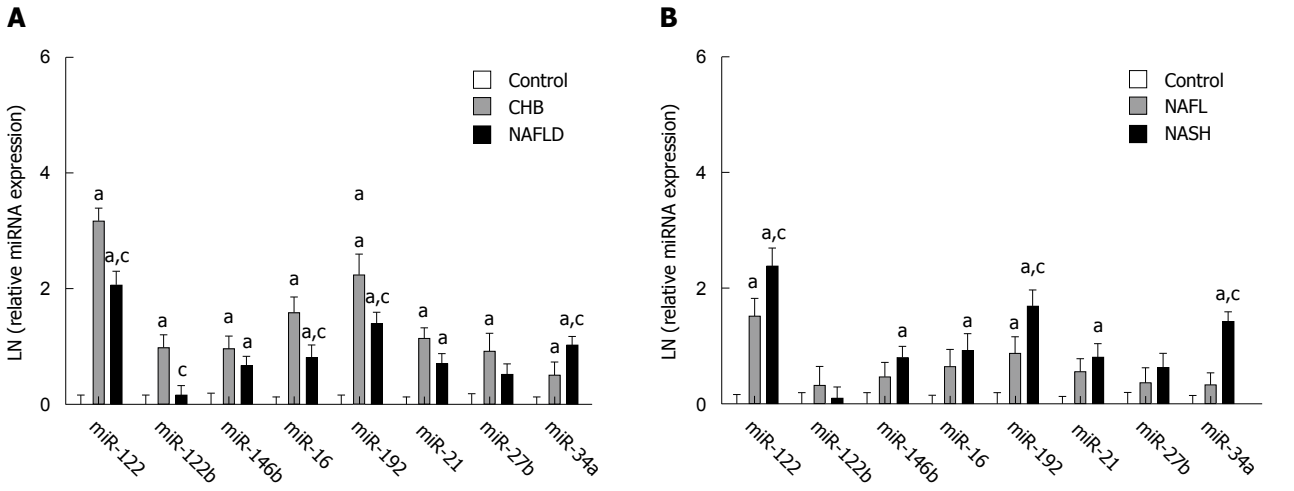


Figure 2 Serum miRNA expression profile among control (A), chronic hepatitis B and non-alcoholic fatty liver disease patients, as well as control (B), non-alcoholic fatty liver and non-alcoholic steatohepatitis patients. All eight miRNAs, except miR-125 and -27b, were up-regulated in the non-alcoholic fatty liver disease (NAFLD) group compared with the control group ($P < 0.05$), with miR-34a being even higher and miR-122, -16 and -192 being lower in the NAFLD group compared with chronic hepatitis B (CHB) group ($P < 0.01$; Figure 2A). Among the miRNAs, miR-122, -192 and -34a showed significant differences not only between the NAFLD and the control groups but also between the non-alcoholic steatohepatitis (NASH) and the non-alcoholic fatty liver (NAFL) groups ($P < 0.001$; Figure 2B). $^{a}P < 0.05$ *vs* the control group; A: $^{c}P < 0.05$ *vs* the CHB group; B: $^{c}P < 0.05$ *vs* the NAFL group.

Table 3 Correlations between miRNAs and histological features

R	miR-122	miR-125b	miR-146b	miR-16	miR-192	miR-21	miR-27b	miR-34a
Steatosis	0.302[1]	0.143	0.230	0.068	0.323[1]	0.199	0.166	0.470[2]
Activity	0.445[2]	0.052	0.218	0.267	0.447[2]	0.320[1]	0.179	0.517[2]
Lobular inflammation	0.285[1]	-0.064	0.137	0.159	0.396[2]	0.254	0.184	0.552[2]
Hepatocellular ballooning	0.477[2]	0.154	0.225	0.290[1]	0.350[1]	0.281	0.101	0.317[1]
Fibrosis	-0.021	-0.078	0.125	0.350[1]	0.145	0.199	-0.028	0.134

Activity is the sum of the score of lobular inflammation and hepatocellular ballooning. [1]$P < 0.05$, [2]$P < 0.01$.

miR-34a significantly increased the sensitivity to 0.704, with a comparable specificity. Compared with miR-34a, FIB-4 had the same sensitivity (0.704) but lower specificity (0.687) for the diagnosis of NASH, and APRI showed the same specificity (0.875) but lower sensitivity (0.556). For the diagnosis of significant fibrosis, miR-16 displayed a relatively lower AUROC (0.716, 95%CI: 0.551-0.882) than that of FIB-4 (0.835, 95%CI: 0.694-0.976) and APRI (0.853, 95%CI: 0.720-0.986; Figure 4B).

DISCUSSION

The past decade has witnessed growing interest in miRNAs for their noninvasive diagnostic value in a variety of diseases, including NAFLD. Previous human studies have examined the miRNA expression pattern in NAFLD, and several miRNAs have been proposed to be predictors of this disease[12-17]. However, there is limited information about the disease specificity and correlation with hepatic histological features of these miRNAs. Therefore, our study explored the serum levels of miR-122, -125b, -146b, -16, -21, -192, -27b and -34a in biopsy-proven NAFLD patients, CHB patients, and healthy controls from mainland China. Among these miRNAs, miR-122, -16, -192 and -34a could differentiate NAFLD from healthy controls and CHB patients, and miR-122, -192 and -34a could distinguish NASH from NAFL. In addition, miR-34a showed a superior ability to diagnose NASH compared with that of ALT, CK-18 (M30) and CK-18 (M65).

Among the eight miRNAs selected from previous articles, miR-122, -16, -192 and -34a showed differential expression in NAFLD and CHB, which are the most common liver diseases in China. Interestingly, only miR-34a was up-regulated in NAFLD compared with CHB patients, indicating that miR-34a may be a more sensitive indicator of NAFLD than CHB. Increased miR-34a levels have been reported in the serum and liver of NAFLD patients and animal models[15,16,21], and our results suggested that miR-34a could distinguish NAFLD not only from healthy controls but also from other liver diseases, such as CHB.

miR-122, a liver-enriched and liver-specific miRNA, has received considerable attention as a biomarker of NAFLD. It is released into circulation during hepatocyte damage and has been shown to be key regulator of lipid metabolism[22]. However, increased miR-122 has also been found in many liver conditions, including hepatitis C virus (HCV) and HBV infections and alcohol-

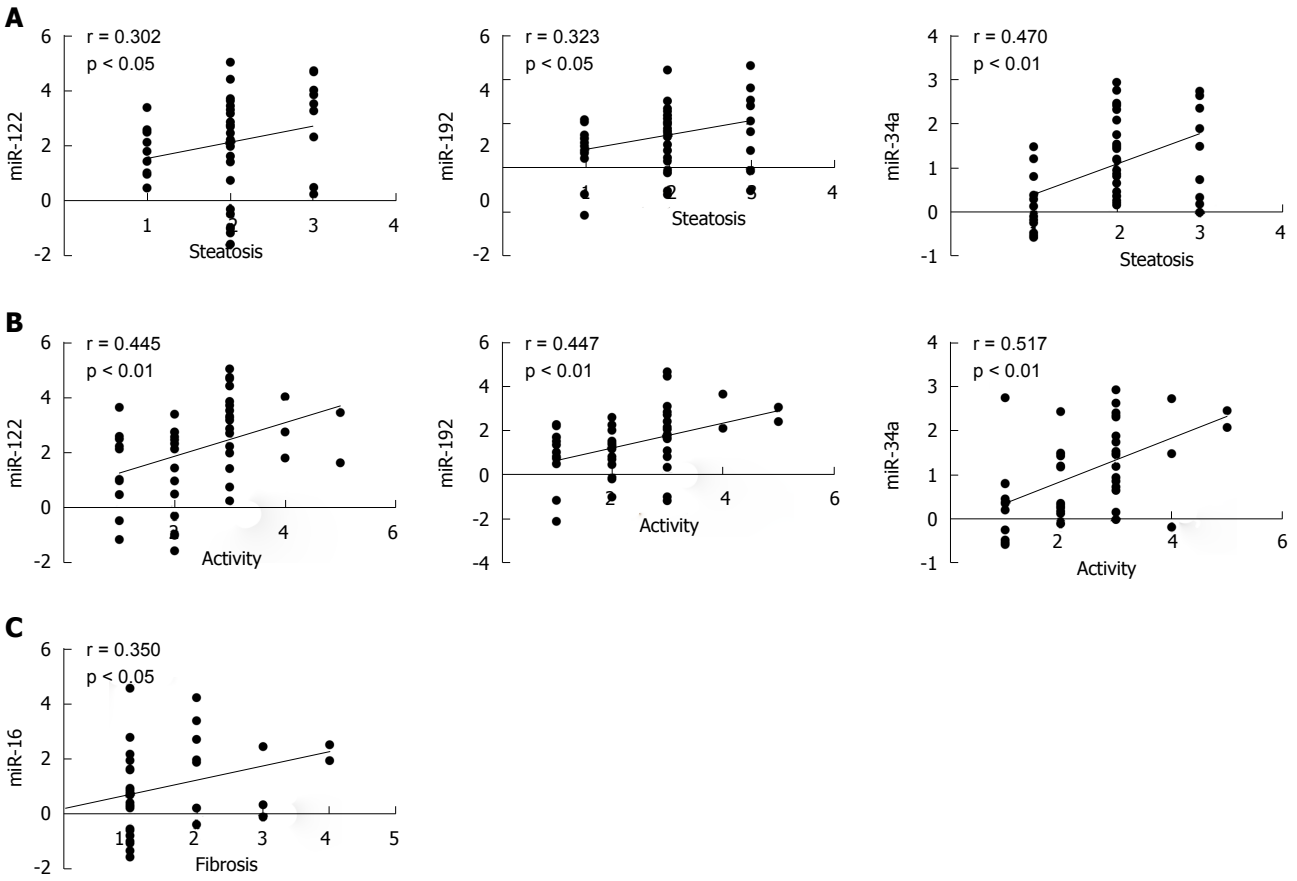


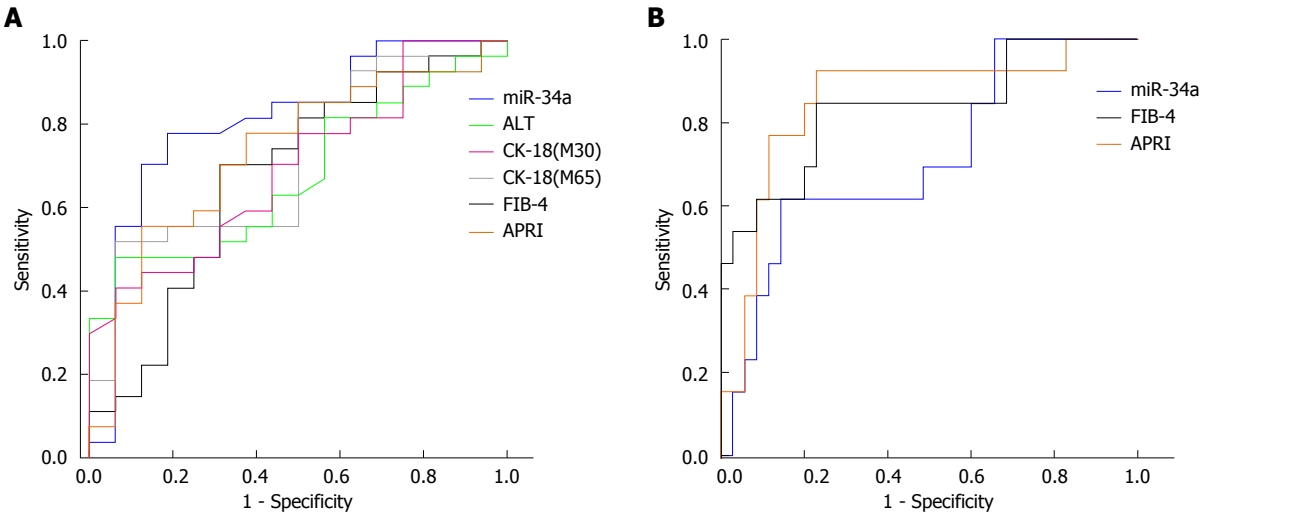
Figure 3 Correlation between circulating miRNAs and hepatic steatosis (A), activity (B) and fibrosis (C) in non-alcoholic fatty liver disease patients. Circulating miR-122, -192 and -34a were correlated with hepatic steatosis ($P < 0.05$) and inflammatory activity ($P < 0.01$). However, only miR-16 was associated with hepatic fibrosis ($P < 0.05$). Activity is the sum of the score of lobular inflammation and hepatocellular ballooning.

and drug-induced liver injury[23-26]. The serum miR-122 levels in CHB patients were much higher than those in NAFLD patients, limiting its value in the diagnosis of NAFLD. Consequently, miR-122 may serve as a marker of general liver damage due to its increased expression in a broad range of liver diseases. In contrast to miR-122, miR-192 has been identified as an oncogene in several cancers, such as colon cancer, breast cancer and gastric cancer[27], and is negatively correlated with the liver metastatic potential of colon cancer cells[28]. Although two independent studies reported increased serum miR-192 levels in NAFLD[12,14], little is known about its function in other liver diseases. Similarly, miR-192 had lower serum levels in NAFLD patients than those of CHB patients, which casts doubt on its diagnostic value in NAFLD.

An intriguing finding in this study is the putative role of serum miR-34a as an extra-hepatic fingerprint of NASH. In recent years, many noninvasive, diagnostic biomarkers have been extensively examined for clinical applications for NASH. CK-18 has potential value in differentiating NASH from NAFL, but consistent with the results by Cusi *et al*[29], it exhibited limited sensitivity in the diagnosis of NASH in our study. Fortunately, miR-34a significantly increased the sensitivity to 0.704, with a specificity of 0.875. Furthermore, our results showed that neither M30 nor M65 could differentiate etiology-related liver diseases. Compared with CK-18, miR-34a not only had a higher AUROC in the diagnosis of NASH but also had good disease specificity. To the best of our knowledge, we report here the first identification of miRNA biomarkers in NASH with regard to their disease specificity.

Our study found a good correlation between hepatic steatosis and serum miR-122, -192 and -34a, especially for miR-34a. Meanwhile, serum miR-34a levels showed the strongest correlation with the severity of hepatic inflammatory activity, including lobular inflammation and hepatocellular ballooning. miR-34a can down-regulate sirtuin 1 (SIRT1), leading to adenosine monophosphate-activated protein kinase (AMPK) dephosphorylation and ultimately cholesterol accumulation[30]. Other studies reported that miR-34a regulates lipoprotein metabolism and promotes liver steatosis and hypolipidemia in a peroxisome proliferator-activated receptor (PPAR) α-dependent manner[31].

There is also a functional link between aberrantly elevated miR-34a and impaired expression of the cytochrome P450 family and the fibroblast growth factor (FGF) family, which indicates the importance of miR-34a in metabolic disorders[32,33]. In addition, the down-regulation of SIRT1 by miR-34a also increased



Parameter	AUROC	95%CI	Sensitivity	Specificity	Youden's index
miR-34a	0.811	0.670-0.953	0.704	0.875	0.579
ALT	0.677	0.518-0.836	0.481	0.937	0.418
CK-18(M30)	0.697	0.539-0.854	0.407	0.937	0.344
CK-18(M60)	0.718	0.560-0.875	0.519	0.937	0.456
FIB-4	0.676	0.503-0.849	0.704	0.687	0.391
APRI	0.727	0.569-0.884	0.556	0.875	0.431

Parameter	AUROC	95%CI	Sensitivity	Specificity	Youden's index
miR-34a	0.716	0.551-0.882	0.615	0.857	0.486
FIB-4	0.835	0.694-0.976	0.846	0.771	0.617
APRI	0.853	0.720-0.986	0.923	0.771	0.694

Figure 4 Receiver operating characteristic curve of miR-34a, alanine aminotransferase, cytokeratin-18, fibrosis-4 and aspartate aminotransferase to platelet ratio index for on-alcoholic steatohepatitis (A) and miR-16, fibrosis-4 and aspartate aminotransferase to platelet ratio index for significant fibrosis (B) diagnosis in non-alcoholic fatty liver disease. The AUROC of miR-34a and miR-16 for non-alcoholic steatohepatitis (NASH) and significant fibrosis were 0.811 and 0.716, respectively. Circulating miR-34a significantly increased the sensitivity to 0.704, with a comparable specificity. For the diagnosis of significant fibrosis, miR-16 displayed a relatively lower AUROC than that of FIB-4 and APRI. ALT: Alanine aminotransferase; CK-18: Cytokeratin-18; FIB-4: Fibrosis-4; APRI: Aspartate aminotransferase to platelet ratio index.

acetylation of p53, thereby activating apoptosis of hepatocytes[34]. CK-18 and miR-34a reflect a common pathophysiological status, a caspase-related apoptosis pathway, which may explain the good correlation between the serum levels of these markers in our study. In contrast to the findings that miR-34a promotes hepatic fibrosis through activation of hepatic stellate cells (HSCs)[15], our study found no positive relationship between serum miR-34a and hepatic fibrosis in NAFLD patients.

Nevertheless, the circulating miR-16 levels were positively correlated with hepatic fibrosis and showed a good ability to diagnose significant fibrosis in NAFLD, although its diagnostic value was slightly lower than that of FIB-4 and APRI. The FIB-4 and APRI were identified as good biomarkers for the presence and severity of hepatic fibrosis in NAFLD patients[35]. The activation of HSCs is generally considered to be the major mechanism responsible for liver fibrosis. Our previous work showed that miR-16 is related to the activation of cultured rat HSCs and likely participates in HSC apoptosis by targeting Bcl-2 and the downstream protease cascade[36].

Additionally, the up-regulation of miR-16 has been shown to contribute to the development of liver fibrosis and HCC in HCV and HBV infections, respectively[37,38]. miR-16 in visceral adipose tissue also showed an increase in obese NASH patients[17], while the data from liver tissues of NAFLD patients are limited. Therefore, it would be interesting to explore the behavior of miR-16 in livers from NASH patients to confirm the specific function of miR-16 in NAFLD in future studies.

Finally, several limitations of this study need to be noted. The subjects enrolled in our study were Han individuals from mainland China, and the results should be validated in other ethnic groups and races with larger samples. The above results did not provide direct evidence for the involvement of these differentially expressed miRNAs in the progression of NAFLD, but they could serve as the basis for functional research on this disease in the future.

In conclusion, our study demonstrated that the serum levels of miR-122, -16, -192 and miR-34a showed differential expression in NAFLD and CHB patients. Circulating miR-34a had a moderate correlation with hepatic steatosis and inflammatory activity and showed a superior ability to diagnose NASH compared to that of ALT and CK-18, which highlights its potential value as a biomarker of disease severity. In addition, circulating miR-16 exhibited a good correlation with hepatic fibrosis but had a slightly poorer performance than that of FIB-4 and APRI in distinguishing

significant fibrosis in NAFLD.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) has become a growing public health concern worldwide. Currently, non-alcoholic steatohepatitis (NASH) is the most common cause of chronic liver disease. Thus, there is an urgent need in the development of noninvasive biomarkers for this disease. In Asia, especially China, chronic hepatitis B (CHB) is still a leading cause of cirrhosis and hepatocellular carcinoma (HCC). Given the high prevalence of CHB in China, it cannot be excluded in the identification of biomarkers of NAFLD and NASH.

Research frontiers

Recently, circulating microRNAs (miRNAs) have emerged as attractive candidate biomarkers for early detection and monitoring of disease progression and response to treatment. The use of miRNAs as noninvasive biomarkers of NAFLD is of particular interest.

Innovations and breakthroughs

Recent studies have identified miR-122, -125b, -146b, -16, -21, -192, -27b and -34a as the most common regulators contributing to the development of NAFLD. However, there is still a lack of reliable disease-specific biomarkers for the diagnosis of NASH. Therefore, we assessed whether circulating miRNAs can distinguish NAFLD from CHB and also diagnose NASH and significant fibrosis in NAFLD.

Applications

The current study identified that the serum levels of miR-122, -16, -192 and miR-34a could differentiate NAFLD from CHB patients, and that miR-122, -192 and miR-34a could differentiate NASH from NAFL. Circulating miR-34a showed a superior ability to diagnose NASH compared to that of alanine aminotransferase and cytokeratin-18, highlighting its potential value as a biomarker of NASH in clinical practice. Meanwhile, the results above could serve as the basis for functional research on this disease in the future. The subjects enrolled in our study were Han individuals from mainland China, so the results should be validated in other ethnic groups and races with larger samples in the future.

Terminology

MiRNAs are short noncoding RNAs composed of 18-25 nucleotides that regulate gene expression at the post-transcriptional level. Each miRNA has numerous target genes, and hundreds of miRNAs can regulate one target gene. MiRNAs are important players in a wide spectrum of biological processes and metabolic homeostasis, including protein secretion and fatty acid metabolism. Meanwhile, circulating miRNAs are protected from RNase in circulation and are extremely stable, so they have emerged as attractive candidate biomarkers for early diagnosis of disease and to monitor disease progression.

Peer-review

This paper by Liu *et al* is an interesting study on a new diagnostic biomarker of NASH. This study examined the performance of a new diagnostic biomarker panel for NASH. They found that serum level of miR-34a correlated with histological features of NASH including steatosis, hepatocyte ballooning and lobular inflammation. This study is well conducted and the methods used are appropriate. The data is presented clearly. These findings will be of interest to [clinical] practitioners as well as researchers in the field.

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ORIGINAL ARTICLE

Prospective study

Hepatitis E virus: Western Cape, South Africa

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Abstract

AIM

To conduct a prospective assessment of anti-hepatitis E virus (HEV) IgG seroprevalence in the Western Cape Province of South Africa in conjunction with evaluating risk factors for exposure.

METHODS

Consenting participants attending clinics and wards of Groote Schuur, Red Cross Children's Hospital and their affiliated teaching hospitals in Cape Town, South Africa, were sampled. Healthy adults attending blood donor clinics were also recruited. Patients with known liver disease were excluded and all major ethnic/race groups were included to broadly represent local demographics. Relevant demographic data was captured at the time of sampling using an interviewer-administered confidential questionnaire. Human immunodeficiency virus (HIV) status was self-disclosed. HEV IgG testing was performed using the Wantai® assay.

RESULTS

HEV is endemic in the region with a seroprevalence of 27.9% (n = 324/1161) 95%CI: 25.3%-30.5% (21.9% when age-adjusted) with no significant differences between ethnic groups or HIV status. Seroprevalence in children is low but rapidly increases in early adulthood. With univariate analysis, age $\geqslant$ 30 years old, pork and bacon/ham consumption suggested risk. In the multivariate analysis, the highest risk factor for HEV IgG seropositivity (OR = 7.679, 95%CI: 5.38-10.96, P < 0.001) was being 30 years or older followed by pork consumption (OR = 2.052, 95%CI: 1.39-3.03, P < 0.001). A recent clinical case demonstrates that HEV genotype 3 may be currently circulating in the Western Cape.

CONCLUSION

Hepatitis E seroprevalence was considerably higher than previously thought suggesting that hepatitis E warrants consideration in any patient presenting with an unexplained hepatitis in the Western Cape, irrespective of travel history, age or ethnicity.

Key words: Hepatitis E; Seroprevalence; South Africa; Pork consumption; Genotype

Core tip: This is a prospective seroprevalence study of 1161 participants assessing anti-hepatitis E virus (HEV) IgG seroprevalence in the Western Cape Province of South Africa. The only risk factors for seropositivity are pork consumption and individuals over 30 years of age. A recent clinical case suggests HEV genotype 3 may be circulating in South Africa.

Madden RG, Wallace S, Sonderup M, Korsman S, Chivese T, Gavine B, Edem A, Govender R, English N, Kaiyamo C, Lutchman O, van der Eijk AA, Pas SD, Webb GW, Palmer J, Goddard E, Wasserman S, Dalton HR, Spearman CW. Hepatitis E virus: Western Cape, South Africa. *World J Gastroenterol* 2016; 22(44): 9853-9859 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9853.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9853

INTRODUCTION

Globally, hepatitis E virus (HEV) is the most frequent aetiological cause of acute hepatitis[1]. It causes sporadic and epidemic infections, predominantly in young adults living in developing countries. In these regions, it is associated with HEV genotypes 1 and 2, which are obligate human pathogens and spread oro-faecally through infected water. Most patients experience a self-limiting hepatitis, except in pregnant women and patients with chronic liver disease, where mortality may reach 25% and 75% respectively[2].

In the developed world, hepatitis E is largely a porcine zoonosis caused by genotypes 3 and 4 and is a cause of self-limiting hepatitis in middle-aged and elderly men[2-4]. Chronic infection occurs in those who are immunosuppressed, including transplant recipients[5], patients with haematological malignancy and individuals with human immunodeficiency virus (HIV)[6]. An important route of infection is through consumption of infected pig meat products in the human food chain[2].

Hepatitis E, invariably genotypes 1 and 2, is seen in a number of African countries. There have been several outbreaks observed in sub-Saharan African refugee camps, including recently South Sudan and Uganda[7,8]. In the 1980's a large outbreak was reported in Namibia where HEV genotype 2 is known to circulate[9]. In South Africa, very few data regarding HEV exists. Two seroprevalence studies from the 1990's demonstrated low rates but these studies were potentially limited as the screening tests employed are now known to have had poor sensitivity[10]. In addition, very few cases of hepatitis E from South Africa have been reported in the literature but recently, two cases caused by HEV genotype 3 have been described[11,12].

Given the paucity of data, we elected to prospectively investigate the seroprevalence of anti-HEV IgG in a South African population in the Western Cape using a sensitive assay as well as assessing risk factors for anti-HEV IgG seropositivity. Unexpectedly, a case of acute hepatitis E infection was documented and is reported here.

MATERIALS AND METHODS

HEV seroprevalence and risk factors

The study was designed to cover all age groups in addition to reflecting the population of the Western

Table 1 Data (%) for total population overall and age group seroprevalence and 95%CI in 3 racial groups in Western Cape, South Africa

Age group (yr)	Total population (n = 1161)		Black African population (n = 389)		MA/Coloured population (n = 452)		White population (n = 320)	
	IgG (n)	HEV Seroprevalence % (95%CI)	IgG (n)	HEV Seroprevalence % (95%CI)	IgG (n)	HEV Seroprevalence % (95%CI)	IgG (n)	HEV Seroprevalence % (95%CI)
0-4	6/72	8.3 (3.9-17.0)	2/36	5.6 (1.5-18.1)	4/31	12.9 (5.1-28.9)	0/5[1]	0 (0-43.4)
5-9	6/78	7.7 (3.6-15.8)	1/26	3.8 (0.7-18.9)	5/45	11.1 (4.8-23.5)	0/7[1]	0 (0-35.4)
10-14	7/72	9.7 (4.8-18.7)	3/27	11.1 (3.9-28.1)	4/42	9.5 (3.8-22.1)	0/3[1]	0 (0-56.2)
15-19	8/95	8.4 (4.3-15.7)	4/23	17.4 (7.0-37.1)	4/42	9.5 (3.8-22.1)	0/30[1]	0 (0-11.4)
20-29	17/179	9.5 (6.0-14.7)	2/62	3.2 (0.9-11.0)	9/54	16.7 (9.0-28.7)	6/63	9.5 (4.4-19.3)
30-39	42/142	29.6 (22.7-37.5)	14/52	26.9 (16.8-40.3)	17/45	37.8 (25.1-52.4)	11/45	24.4 (14.2-38.7)
40-49	67/153	43.8 (36.2-51.7)	22/62	35.5 (24.7-47.9)	22/49	44.9 (31.9-58.7)	23/42	54.8 (39.9-68.8)
50-59	62/126	49.2 (40.6-57.8)	15/36	41.7 (27.1-57.8)	26/47	55.3 (41.2-68.6)	21/43	48.8 (34.6-63.2)
60-69	67/152	44.1 (36.4-52.0)	24/43	55.8 (41.1-69.6)	19/58	32.8 (22.1-45.6)	24/51	47.1 (34.1-60.5)
70-79	34/68	50.0 (38.4-61.6)	6/17	35.3 (17.3-58.7)	16/29	55.2 (37.5-71.6)	12/22	54.5 (34.7-73.1)
80+	8/24	33.3 (18.0-53.3)	1/5	20.0 (3.6-62.4)	2/10	20.0 (5.7-51.0)	5/9	55.6 (26.7-81.1)
Total	324/1161	27.9 (25.3-30.5)	94/389	24.2 (20.2-28.7)	128/452	28.3 (24.4-32.6)	102/320	31.9 (27.0-37.2)

Overall seroprevalence was 27.9% and age adjusted seroprevalence was 21.9%. [1]Numbers too small to calculate a meaningful confidence interval. HEV: Hepatitis E virus.

Cape in terms of ethnic distribution. Participants were asked to self-identify their ethnicity and were randomly recruited and sampled from the three major ethnic groups aged 0 to > 60 years old from both a hospital and non-hospital setting. Participants were recruited from the general medical and Emergency Unit inpatients and outpatients of Groote Schuur, Red Cross Childrens, New Somerset and UCT Private Academic Hospitals between 28/02/14-12/02/2015. Furthermore, healthy blood donors, prior to screening, from two blood donation centres in Cape Town, South Africa were included. Participants with known or reported liver disease were excluded. Following informed consent, blood samples were drawn and each participant completed a structured questionnaire for demographic and known risk factors for hepatitis E acquisition, these included, consumption of pork, sausage, bacon/ham, fish and shellfish; type of dwelling (formal dwelling or informal dwelling/shack in back yard, informal dwelling/shack not in back yard, other); access to piped water (piped water inside dwelling, piped water inside yard, piped water outside yard, no access to piped water); proximity to coast (coastal, < 5 km, 5-10 km, > 10 km) and refuse disposal (removed by local authority/private company, communal refuse dump, own refuse dump, no rubbish disposal/other). Given the massive upscaling of HIV testing in South Africa, participants were asked to self-disclose their HIV status, if known. Blood samples were stored at -70℃ and tested in batches for HEV anti-IgG using the Wantai® assay (Beijing, China) according to the manufacturer's instructions.

Statistical analysis

Age-standardized seroprevalence was calculated using the 2014 South Africa Mid-Year population estimates as the reference population[13]. Age-specific percentages were applied to the South African population, and then summed to determine the age-standardized seroprevalence for anti-HEV IgG in the population as a whole as well as between different ethnic groups. Continuous variables are described using counts and percentages and independent risk factors were explored by uni-variate binary logistic regression. Factors, which were found to be statistically significant on uni-variate analysis, were then modelled with multi-variate binary logistic regression using maximum-likelihood estimation with parameters (0, 1), analysed with IBM SPSS V22.0. Statistical significance was accepted if $P < 0.05$.

Clinical case

A documented case of acute hepatitis E presenting to New Somerset hospital is described; the clinical course and laboratory findings are recorded.

Ethics

Ethics approval was granted by the Faculty of Health Sciences Human Research Ethics Committee of the University of Cape Town. Patients ⩾ 18 years provided consent whilst parents/guardians provided permission/consent for minors. Adolescents provided assent.

RESULTS

About 1161 participants were included in the study with the 3 major ethnic groups in the Western Cape viz. Black Africans (n = 392, 33.5%), Mixed Ancestry (n = 455, 38.9%) and Whites (n = 322, 27.5%), proportionally sampled. The mean age of the population sampled was 36.4 years (SD 22.3) and 53.3% were male. Notably, Whites < 16 years and Black Africans > 60 years of age were relatively under-represented due to low attendance at sampling locations (Table 1 and Figure 1).

The overall anti-HEV IgG seroprevalence was 27.9% (n = 324/1161, 95%CI: 25.3-30.5) and the age adjusted seroprevalence was 21.9%. Seroprevalence in children (< 19 years old) was approximately

Table 2 Risk factor analysis

Potential risk factors (n = 131)	Univariate analyses			
	Groups	n (IgG%)	P value	OR (95%CI)
Race (n = 161)	Black African (n = 389)	94 (24.2%)	0.073	-
	MA/Coloured (n = 452)	128 (28.3%)		1.24 (0.91-1.69)
	White (n = 320)	102 (31.9%)		1.47 (1.06-2.04)
Gender (n = 161)	Male (n = 541)	161 (29.8%)	0.189	-
	Female (n = 620)	163 (26.3%)		0.842 (0.65-1.09)
Pork (n = 131)	Yes (n = 690)	228 (33.0%)	< 0.001	1.93 (1.45-2.55)
	No (n = 441)	90 (20.4%)		-
Sausage (n = 131)	Yes (n = 853)	248 (29.1%)	0.210	1.22 (0.90-1.66)
	No (n = 278)	70 (25.2%)		-
Bacon/Ham (n = 131)	Yes (n = 707)	220 (31.1%)	0.004	1.50 (1.14-1.98)
	No (n = 424)	98 (23.1%)		-
Fish (n = 131)	Yes (n = 1055)	299 (28.3%)	0.532	1.19 (0.69-2.03)
	No (n = 76)	19 (25.0%)		
Shellfish (n = 131)	Yes (n = 548)	163 (29.7%)	0.238	1.17 (0.90-1.52)
	No (n = 583)	155 (26.6%)		-
Type of dwelling (n = 161)	Formal Dwelling (n = 1050)	296 (28.2%)	0.642	-
	Shack in Yard (n = 88)	21 (24.0%)		0.80 (0.48-1.40)
	Shack not in Yard (n = 19)	5 (26.3%)		0.91 (0.33-2.55)
	Other (n = 4)	2 (50.0%)		2.55 (0.36-18.17)
Water access (n = 1161)	Piped dwelling (n = 1041)	292 (28.0%)	0.516	-
	Piped yard (n = 68)	17 (25.0%)		0.855 (0.49-1.51)
	Community tap (n = 44)	11 (25.0%)		0.855 (0.43-1.71)
	No water access (n = 8)	4 (50.0%)		2.565 (0.64-10.32)
Proximity to coast (n = 1160)	Coastal (n = 114)	32 (28.0%)	0.935	-
	Under 5 km (n = 209)	62 (29.7%)		1.081 (0.65-1.79)
	5-10 km (n = 313)	85 (27.2%)		0.955 (0.59-1.54)
	Over 10 km (n = 524)	145 (27.7%)		0.980 (0.62-1.54)
Refuse disposal (n = 1160)	Local authority (n = 1124)	315 (28.0%)	0.725	-
	Communal dump (n = 20)	5 (25.0%)		0.856 (0.31-2.40)
	Own dump (n = 8)	3 (37.5%)		1.541 (0.37-6.50)
	No rubbish disposal (n = 8)	1 (12.5%)		0.367 (0.05-3.00)
HIV status (n = 891)	Positive (n = 60)	14 (23.3%)	0.340	1.35 (0.73-2.50)
	Negative (n = 831)	242 (29.1%)		-
Age group (n = 1156)	< 30 yr (n = 496)	44 (8.9%)	< 0.001	-
	⩾ 30 yr (n = 660)	280 (42.4%)		7.569 (5.36-10.70)
Multivariate analysis (n = 1126)				
Pork	Yes (n = 686)	228 (33.2%)	< 0.001	7.679 (5.38-10.96)
Age group	⩾ 30 yr (n = 660)	276 (42.3%)	< 0.001	2.021 (1.50-2.73)

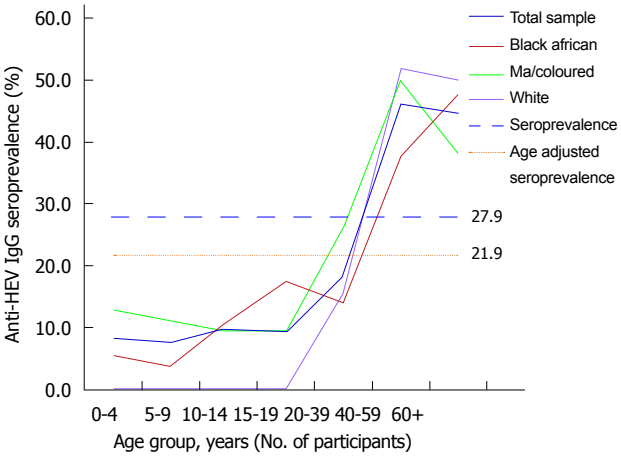

Figure 1 Overall seroprevalence curve and individual seroprevalence curves in 3 racial groups in Western Cape, South Africa. Anti-HEV IgG seroprevalence by age in Western Cape, South Africa.

10% with a rapid increase in seroprevalence in individuals older than 20 years of age (Figure 1). Various demographic and environmental factors were explored with univariate analysis, age, ethnicity, gender, HIV status, consumption of pork, sausages, bacon/ham, fish, shellfish, type of dwelling, access to water, proximity to the coast and method of refuse disposal (Table 2). Age group ⩾ 30 years (P < 0.001), pork consumption (P < 0.001) and bacon/ham consumption (P = 0.004) were strongly associated with a positive HEV IgG. However as the pork and bacon/ham are not independent from one another, only pork consumption was used in the multivariate analysis along with age group (< 30 years, ⩾ 30 years).

The model was statistically significant (P < 0.001) and therefore was able to determine the risk factors of people presenting with HEV IgG positive compared to those that were not and explained between 15%-22% of the variance . With pork in their diet, an individual had an increased risk (OR = 2.02, 95%CI: 1.5-2.73) of being HEV IgG positive compared to those who did not have pork in their diet. Individuals 30 years old or over had an increased risk (OR = 7.679, 95%CI: 5.38-10.96, P < 0.001) of being HEV IgG positive compared to those under 30 years old. No other

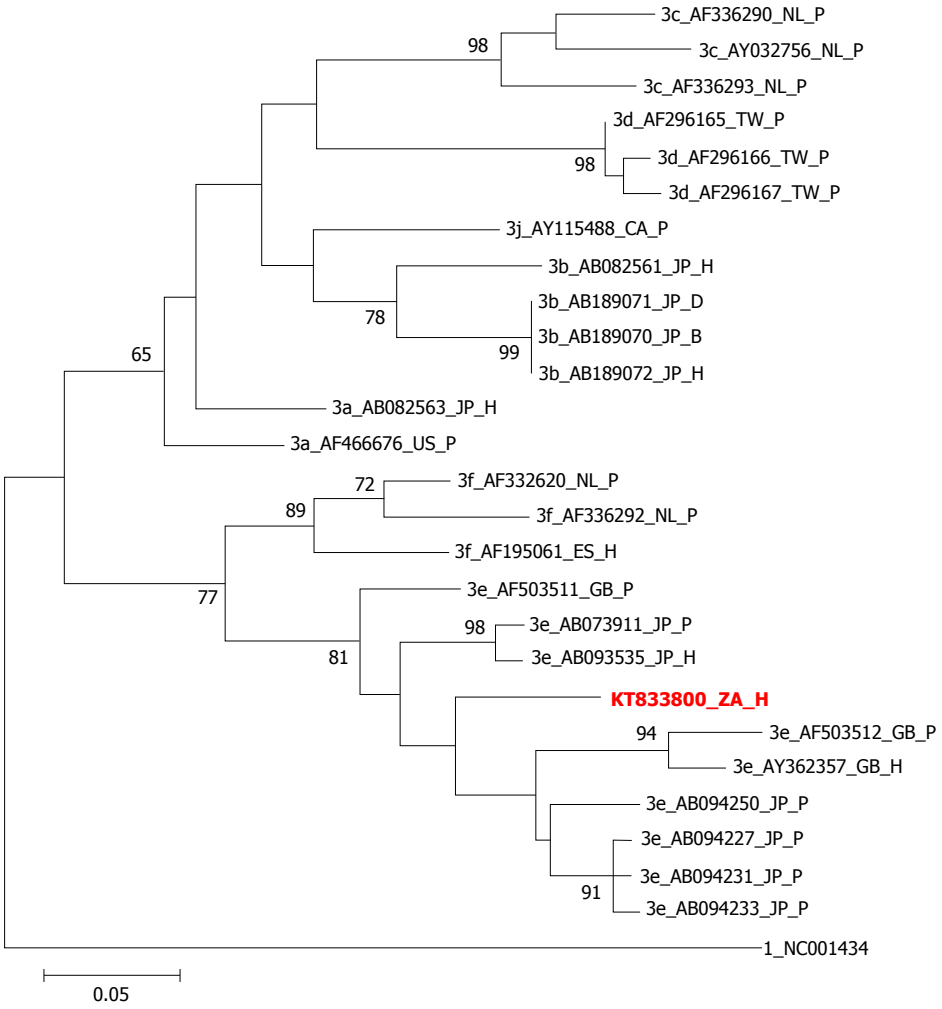

Figure 2 A maximum likelihood tree constructed in MEGA6 from an alignment of a 301nt fragment of ORF2. Bootstrap support above 60% is shown. Our patient's viral sequence, GenBank accession KT833800, is highlighted in red. The tree is rooted on the HEV reference sequence (genotype 1). Sequences for comparison have names starting with genotype, followed by Genbank accession number, followed by country ISO 3166-1 abbreviation (CA: Canada; ES: Spain; GB: United Kingdom; JP: Japan; NL: The Netherlands; TW: Taiwan; US: United States of America; ZA: South Africa), and ending in source (D: Deer; H: Human; P: Pig). The patient's viral sequence clusters within genotype 3 (with subgenotype 3e), in keeping with other viruses recently described in South Africa[11,12,24].

risk factors were associated with HEV seropositivity (Table 2) and therefore excluded from the multivariate analysis.

Clinical case of hepatitis E

In January 2014, a 54-year-old white male presented to New Somerset Hospital, Cape Town, with acute liver failure secondary to a severe hepatitis. Initial clinical examination revealed marked icterus, mild abdominal ascites and smooth non-tender hepatomegaly, as well as signs of chronic liver disease including palmar erythema and spider telangiectasia on the chest wall. The patient worked as a truck driver but had no history of recent travel (in the last 3 mo prior to presentation) outside of the Western Cape. He was not known to have any medical co-morbidities but reportedly consumed significant alcohol. At presentation, blood tests demonstrated (normal range in parentheses): Bilirubin 407 μmol/L (0-18 μmol/L), albumin 28 g/L (35-52 g/L), alanine aminotransferase (ALT) 3054 IU/L (0-40 IU/L), prothrombin time-International normalized ratio (PT-INR) 2.74. Serology for hepatitis A, B, C and HIV were negative. Despite maximal supportive care he demised on day 3 following admission as a result of fulminant hepatic failure. On the day of his death, hepatitis E IgM test returned positive and subsequently HEV RNA genotype 3e was confirmed by polymerase chain reaction (PCR) (see Figure 2).

DISCUSSION

The seroprevalence in Western Cape is much higher than has been previously described in South African populations[10,14]. We found that the overall seroprevalence was 27.9% (21.9% when age-adjusted) and was similar between genders, ethnic groups and HIV status. The age-related seroprevalence curve shows that HEV IgG seropositivity increases with age, but in a non-linear fashion. Seroprevalence is very low in children and young adults, rapidly increasing between

the ages of 20 and 30 years and then plateaus off. Multivariate risk factor analysis demonstarted that the only other significant association with anti-HEV IgG seropositivity was the consumption of pork.

Two previous studies from South Africa in the 1990's described seroprevalence rates of 10.7% and 1.8%-2.6%, respectively. The overall seroprevalence in the current study was an order of magnitude higher. The reason for this difference could relate to geographical location, as both the above previous studies were performed in different areas of South Africa and it is known that HEV seroprevalence can vary enormously between regional areas within countries[15]. Another possible explanation is that there has been an increase in the exposure rates of hepatitis E in South Africa over the last 20 years, which may include changes in migration, diet and enzootic disease. However, the likely dominant explanation relates to the sensitivity of the IgG assays employed in the previous studies. The first generation of anti-HEV IgG assays used in studies in the 1990's lacked sensitivity and generally reported spuriously low seroprevalence rates[16]. In contrast, the assay employed in our study has a sensitivity of 98% at detecting previous infection and produces much higher estimates of seroprevalence[17,18]. However, in common with all currently available anti-HEV IgG assays, the specificity of the Wantai® assay for detecting previous infection has not been fully established although indirect evidence suggests that it has an acceptable specificity. This includes very low seroprevalence rates in young children (in the current study and others from Nepal, Bangladesh and France)[19] and adult populations (Fiji 2%[20], New Zealand 4%[21] and Scotland 4.6%[22])[19-21]. Hence, the high seroprevalence rate reported in our study is probably due to the high sensitivity of the assay used.

The shape of the anti-HEV seroprevalence curve in Western Cape (low rates in childhood, with a rapid increase in early adulthood) is similar to that seen in hyperendemic developing countries such as Nepal and Bangladesh, where HEV genotype 1 predominates[18]. However, the risk factor analysis showed that only pork consumption was significantly associated with HEV seropositivity which is typical of European populations with high incidences of locally-acquired porcine HEV genotype 3[3,15,23]. This finding contrasts with previous data from a different area of South Africa, which showed that rural mud hut dwellers with no access to clean water had higher seropositivity[13]. Moreover, there have been no outbreaks of hepatitis E reported in South Africa which would also argue against genotype 1 possibly being the dominant circulating genotype. Our clinical case, in addition to others, suggests that HEV genotype 3 may be currently circulating in Western Cape. The other cases include two of HEV genotype 3 infection in immunosuppressed patients documented in Cape Town[11,12].

The strengths of the current study are that a highly sensitive, partially validated assay was employed to estimate seroprevalence and that the study population comprised of large numbers of individuals, including adults and children, from all racial groups. The main limitation of the study is that the population was a heterogeneous "convenience population" comprising hospital in-patients, outpatients and healthy blood donors and may not be representative of the population of Western Cape as a whole. The population studied may have over or under-represented certain population subgroups. This includes white children and elderly black South Africans, very few of whom were included in the study.

In conclusion, HEV is endemic in the Western Cape, South Africa with seroprevalence of 27.9%. Seroprevalence in children is very low with a rapid increase in early adulthood. The only risk factors for seropositivity are pork consumption and individuals over 30 years of age. Recent cases suggest HEV genotype 3 may be currently circulating in South Africa. The diagnosis of hepatitis E should be considered in any patient presenting with hepatitis in Western Cape, irrespective of travel history, age or ethnicity.

COMMENTS

Background

Globally, hepatitis E virus (HEV) is the most frequent aetiological cause of acute hepatitis. It causes sporadic and epidemic infections, predominantly in young adults living in developing countries. In these regions, it is associated with HEV genotypes 1 and 2, which are obligate human pathogens and spread oro-faecally through infected water. Most patients experience a self-limiting hepatitis, except in pregnant women and patients with chronic liver disease, where mortality may reach 25% and 75% respectively.

Research frontiers

Many previous seroprevalanece studies in HEV employed poor assays and have in due course underestimated the extent of the problem in these geographical settings. The authors used a highly sensitive and specific assay; Wantai® demonstrating a seroprevalence of 27.9%. It also demonstrated a potential circulating gentotype previously thought not to be circulating in this particular area: HEV3.

Innovations and breakthroughs

Only pork consumption was significantly associated with HEV seropositivity which is typical of European populations with high incidences of locally-acquired porcine HEV genotype 3. This finding contrasts with previous data from a different area of South Africa, which showed that rural mud hut dwellers with no access to clean water had higher seropositivity. Moreover, there have been no outbreaks of hepatitis E reported in South Africa which would also argue against genotype 1 possibly being the dominant circulating genotype.

Applications

The diagnosis of hepatitis E should be considered in any patient presenting with hepatitis in Western Cape, irrespective of travel history, age or ethnicity.

Peer-review

This study describes the prevalence of anti-HEV IgG in hospital patients and blood donors in Western Cape, South Africa, and identified consumption of pork as its risk factor. The study also reported a case of fulminant hepatic failure following acute HEV infection with genotype 3 in a patient who had chronic alcoholic liver disease. The study provides useful information for better

understanding of HEV epidemiology in South Africa.

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CASE REPORT

Spontaneous rupture of hepatic metastasis from a thymoma: A case report

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Author contributions: Park YE, Lee SJ and Park YN analyzed the data; Kim HJ, Ki MS, Beom SH, Han DH and Park JY treated the patient; Kim HJ and Park JY wrote the paper.

Institutional review board statement: This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the institutional review board of Severance Hospital. A written informed consent was not required due to the retrospective nature of this study.

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Abstract

Bleeding resulting from spontaneous rupture of the liver is an infrequent but potentially life threatening complication that may be associated with an underlying liver disease. A hepatocellular carcinoma or hepatic adenoma is frequently reported is such cases. However, hemoperitoneum resulting from a hepatic metastatic thymoma is extremely rare. Here, we present a case of a 62-year-old man with hypovolemic shock induced by ruptured hepatic metastasis from a thymoma. At the first hospital admission, the patient had a 45-mm anterior mediastinal mass that was eventually diagnosed as a type A thymoma. The mass was excised, and the patient was disease-free for 6 years. He experienced sudden-onset right upper quadrant pain and was again admitted to our hospital. We noted large hemoperitoneum with a 10-cm encapsulated mass in S5/8 and a 2.3-cm nodular lesion in the right upper quadrant of the abdomen. He was diagnosed with hepatic metastasis from the thymoma, and he underwent chemotherapy and surgical excision.

Key words: Spontaneous rupture; Hemoperitoneum; Transarterial chemoembolization; Hepatic metastasis; Thymoma

Core tip: Spontaneous rupture of hepatic metastasis from a thymoma is extremely rare. This case report presents a rare case of spontaneous rupture of hepatic metastasis from a thymoma and describes its management. To our knowledge, no such case of rupture of a metastatic liver tumor associated with a thymoma has been reported previously.

Kim HJ, Park YE, Ki MS, Lee SJ, Beom SH, Han DH, Park YN, Park JY. Spontaneous rupture of hepatic metastasis from a thymoma: A case report. *World J Gastroenterol* 2016; 22(44): 9860-9864 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9860.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9860

INTRODUCTION

The most common cause of a tumor in the anterior mediastinum is a thymic tumor. They have the ability to invade local structures and the capacity to metastasize to distant organs. The sites of metastasis include the lymph nodes, lungs, bones, liver, spleen, brain, and adrenal glands. However, hepatic metastasis is uncommon, and it is extremely rare for a metastatic liver tumor to rupture and cause hemoperitoneum. Recently, we experienced a patient who underwent transarterial chemoembolization for spontaneous rupture of hepatic metastasis from a thymoma. Here, we present this extremely rare case. To our knowledge, no such case of rupture of a metastatic liver tumor associated with a thymoma has been reported previously.

CASE REPORT

A 62-year-old man underwent an annual health checkup. A chest radiogram obtained during the checkup showed widening of the mediastinum. Chest computed tomography showed a 45-mm anterior mediastinal mass (Figure 1), and he underwent excision of the mass. A biopsy specimen from the mass demonstrated that the tumor was a type A thymoma according to the World Health Organization classification system, with fat tissue invasion. The tumor included bland spindle cells and few lymphocytes (Figure 2). According to the Masaoka classification system, the tumor stage was IIA, and he received radiotherapy (total 50 Gy in 25 fractions).

He was disease-free for 6 years; however, he experienced sudden-onset right upper quadrant pain and was referred to our hospital. He did not have a history of abdominal trauma and did not experience fever, vomiting, or diarrhea. On examination, his blood pressure was 77/50 mmHg and pulse rate was 70 beats/min. Blood analysis revealed a white blood cell count of 15370/mm^3 (reference range, 4000-10800/mm^3), hemoglobin level of 10.5 g/dL (reference range, 13-17.4 g/dL), platelet count of 154000/mm^3 (reference range, 150000-400000/mm^3), C-reactive protein level of 6 mg/L (reference range, 0-8 mg/L), aspartate aminotransferase level of 19 IU/L (reference range, 13-34 IU/L), alanine aminotransferase level of 17 IU/L (reference range, 5-46 IU/L), and alkaline phosphatase level of 40 IU/L (reference range, 47-143 IU/L). Abdominal computed tomography revealed large hemoperitoneum with a 10-cm encapsulated mass in S5/8 and a 2.3-cm nodular lesion in the right upper quadrant of the abdomen (Figure 3). The tumor marker (α-fetoprotein and prothrombin induced by vitamin K absence-II) levels were within the reference limits (2.3 ng/mL and 10 mAU/mL, respectively). Based on these findings, he underwent emergency angiography, and an interventional radiologist embolized the vessel supplying the tumor (Figure 4). Five days after embolization, when the patient was stable, abdominal wall biopsy was performed under general anesthesia. Histopathological analysis indicated a metastatic type A thymoma (Figure 5). He was transferred to the oncology department and underwent chemotherapy with cyclophosphamide (500 mg/m^2 day 1), doxorubicin (50 mg/m^2 day 1), and cisplatin (50 mg/m^2 day 1). After 4 cycles of chemotherapy, segmentectomy of the hepatic metastasis was performed and liver biopsy showed histopathology similar to a thymoma.

DISCUSSION

Spontaneous rupture of hepatic tumors usually occurs without warning in patients with hepatocellular carcinoma (HCC), and it is regarded as one of the most serious conditions associated with HCC[1]. However, hemoperitoneum induced by rupture of a metastatic liver tumor is extremely. Ruptured metastatic liver tumors originating from various cancers, such as cancers of the lung, breast, testicle, gallbladder, stomach, kidney, skin, and nasopharynx; choriocarcinoma; and hepatic lymphoma, have been reported[2]; however, to our knowledge, this is the first report to describe a ruptured metastatic liver tumor that originated from a thymoma.

Thymic tumors are derived from thymic epithelial cells, and are located mainly in the anterior mediastinum. About 90% of thymic tumors are located in the anterior mediastinum and some are found in other mediastinal areas. However, the incidence of thymoma is very low, and has been shown to be 0.15 cases per 100000 population. The disease progression is very slow, the condition remains silent for many years, and the tumor is generally incidentally

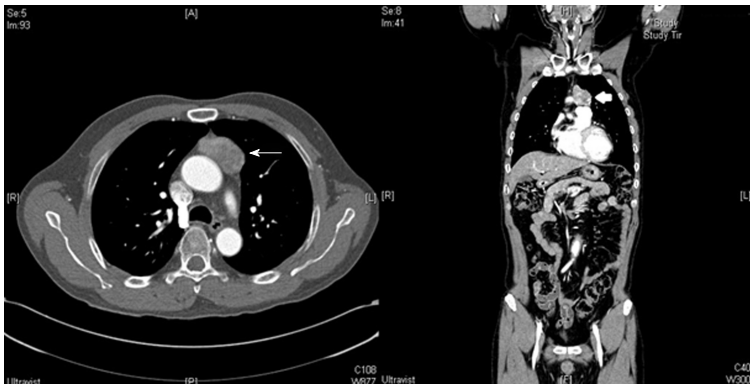

Figure 1 Computed tomography images. A mass (maximum diameter, 45 mm; white arrow) is seen anterior to the ascending thoracic aorta. Histological analysis later identified the mass as a thymoma.

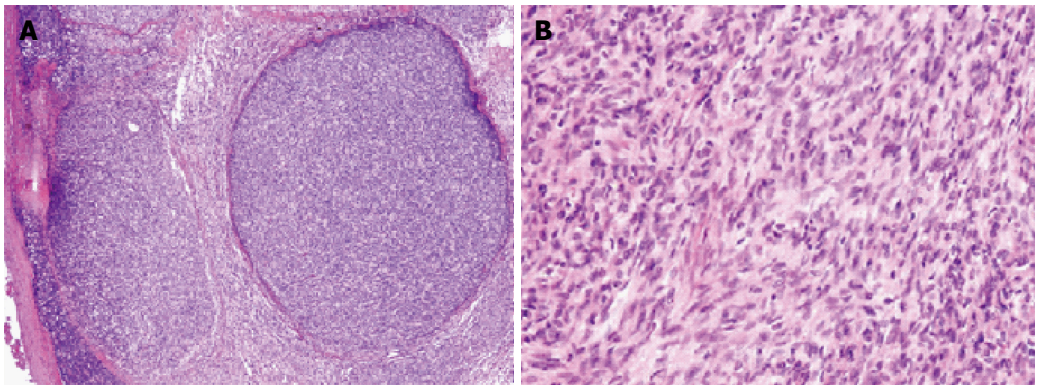

Figure 2 Histological analysis of the mediastinal thymoma. A: A stained section of the mediastinal thymoma (hematoxylin-eosin staining, magnification × 40); B: The type A component is composed primarily of fibroblast-like spindle cells (hematoxylin-eosin staining, magnification × 100).

identified[3]. A multidisciplinary approach is essential in the management of a thymoma. If complete resection of the primary cancer appears possible, surgery is an important treatment approach for thymic tumors. Adjuvant radiotherapy has been widely used to improve survival or reduce the tumor in cases of late-stage cancer, especially when the tumor penetrates adjacent tissues or when the tumor is incompletely resected. Systemic chemotherapy is currently considered as the standard treatment approach for metastatic or inoperable tumors[4].

A thymoma is capable of both local invasion and intrathoracic recurrence, and local recurrence is common. The sites of metastasis include the brain, spleen, adrenal glands, bones, lymph nodes, liver, and lungs. The lung is considered the most frequent location of distant metastasis, while the liver has a relatively lower incidence of metastasis, and hepatic metastasis represents 12.5%-33.5% of all metastatic cases[3]. Hemoperitoneum originating from a metastatic thymoma is extremely rare.

Treatment of hemoperitoneum secondary to hepatic metastasis depends on tumor location, tumor size, and bleeding severity. The primary goal should be complete hemostasis[5]. There are different treatment options for patients presenting with hepatic rupture. These treatment options vary from conservative management to more invasive procedures, such as hepatectomy and transarterial embolization (TAE). A previous study compared different treatment options for hepatic rupture and concluded that the survival rate is better with hepatectomy than with TAE[6]. Although hepatic resection may be a definitive treatment for bleeding, most patients have impaired liver function and an unstable hemodynamic status, and therefore, cannot tolerate surgery. In such cases, TAE and conservative management have been used to control bleeding after hepatic rupture[7]. With regard to HCC rupture, the risk of mortality is higher with conservative management than with TAE. Thus, in emergencies, TAE is frequently performed as initial therapy. Hemoperitoneum secondary to hepatic metastasis from a choriocarcinoma and a gastrointestinal stromal tumor has been successfully treated with TAE[2,8]. However, care should be taken, as artery embolization is associated with complications, such as re-bleeding and

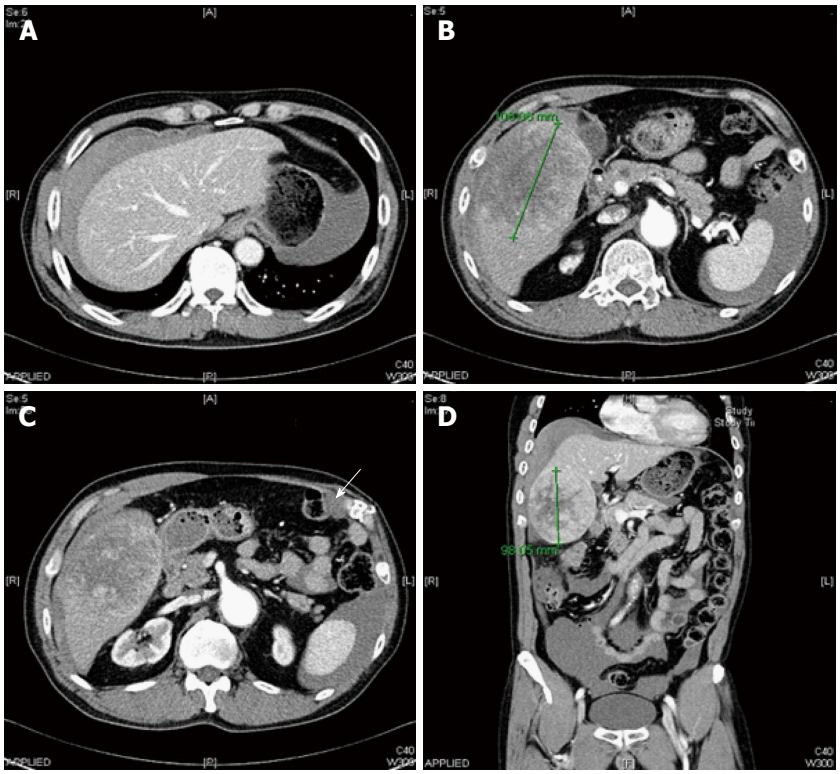

Figure 3 Enhanced computed tomography images. A: Large hemoperitoneum is seen around the liver; B: A 10-cm encapsulated mass is seen in S5 and S8 of the liver, and hemoperitoneum is seen around the spleen; C: A 2.3-cm nodular lesion is seen in the right upper quadrant of the abdomen adjacent to the left transverse abdominal muscle (white arrow); D: Coronal view of the large hemoperitoneum with a liver mass.

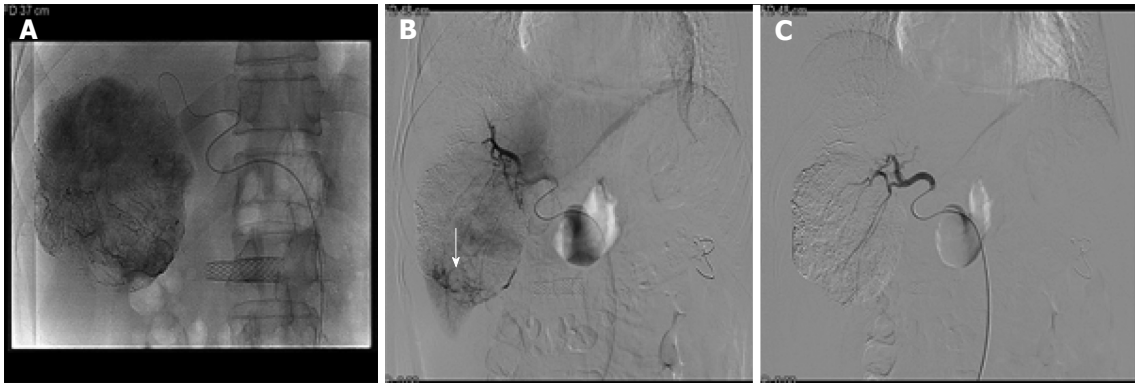

Figure 4 Angiography images. A: Digital subtraction angiography shows a large ruptured hypervascular tumor with staining at the right hepatic lobe during transarterial embolization; B: Active contrast leakage (white arrow) is seen; C: Selection of the right hepatic artery with a mixture of adriamycin (50 mg) and lipiodol (20 mL). The tumor staining disappears after transarterial embolization.

peritoneal seeding. Massive peritoneal washing with saline solution has been shown to reduce the chance of peritoneal implantation[9]. Additionally, to reduce the chance of recurrent bleeding, TAE can be used as a bridge to hepatectomy. A previous report showed the favorable outcomes of delayed hepatectomy after emergency transcatheter arterial embolization for hepatic rupture[10]. Additionally, in a previous study, patients treated with staged hepatectomy had the highest survival rate[1]. In our case, we managed the ruptured hepatic tumor with arterial embolization owing to hypovolemic shock. Complete hemostasis was achieved, and delayed hepatectomy was performed for the hepatic metastasis.

In conclusion, we presented a rare case of spontaneous rupture of hepatic metastasis from a thymoma

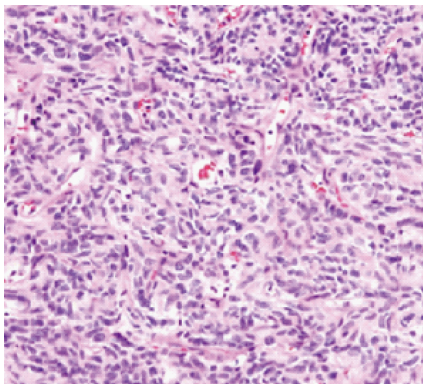

Figure 5 Histopathological analysis of the abdominal wall mass. The tumor is composed of fibroblast-like spindle cells (thymoma type A, metastatic).

and described its management.

COMMENTS

Case characteristics

A 62-year-old Asian man with a thymoma visited the emergency room after experiencing whole abdominal pain.

Clinical diagnosis

Hemoperitoneum.

Differential diagnosis

Septic shock due to pan-peritonitis.

Laboratory diagnosis

The hemoglobin level was 10.5 g/dL, and tumor markers (α-fetoprotein and prothrombin induced by vitamin K absence-II) were negative.

Imaging diagnosis

Abdominal computed tomography revealed large hemoperitoneum with a 10-cm encapsulated mass in S5/8 and a 2.3-cm nodular lesion in the right upper quadrant of the abdomen.

Pathological diagnosis

The thymoma and metastatic liver tumor were composed of fibroblast-like spindle cells.

Treatment

Transarterial embolization (TAE) and delayed hepatectomy were performed.

Experiences and lessons

TAE is an excellent treatment option for hemoperitoneum secondary to hepatic metastasis from a thymoma.

Peer-review

No such case of rupture of a metastatic liver tumor associated with a thymoma has been reported previously.

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CASE REPORT

Pediatric living donor liver transplantation for congenital hepatic fibrosis using a mother's graft with von Meyenburg complex: A case report

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Abstract

This is the first report of living donor liver transplantation (LDLT) for congenital hepatic fibrosis (CHF) using a mother's graft with von Meyenburg complex. A 6-year-old girl with CHF, who suffered from recurrent gastrointestinal bleeding, was referred to our hospital for liver transplantation. Her 38-year-old mother was investigated as a living donor and multiple biliary hamartoma were seen on her computed tomography and magnetic resonance imaging scan. The mother's liver function tests were normal and she did not have any organ abnormality, including polycystic kidney disease. LDLT using the left lateral segment (LLS) graft from the donor was performed. The donor LLS graft weighed 250 g; the graft recipient weight ratio was 1.19%. The operation and post-operative course of the donor were uneventful and she was discharged on post-operative day (POD) 8. The graft liver function was good, and the recipient was discharged on POD 31. LDLT using a graft with von Meyenburg complex is safe and useful. Long-term follow-up is needed with respect to graft liver function and screening malignant tumors.

Key words: Congenital hepatic fibrosis; Von Meyenburg complex; Living donor liver transplantation; Pediatric liver transplantation

Core tip: Multiple biliary hamartoma is a rare, benign lesion known as von Meyenburg complex. This is the first report of living donor liver transplantation (LDLT) using a liver graft with von Meyenburg complex. A 6-year-old girl with congenital hepatic fibrosis, who suffered from recurrent gastrointestinal bleeding, was transplanted her mother's liver graft with von Meyenburg complex. Successful LDLT was performed, and the liver and renal function after LDLT were good also in recipient and donor. LDLT using a graft with von Meyenburg complex is safe and useful for a further expansion of living donor pool. Long-term follow-up is needed with respect to graft liver function and screening malignant tumors.

Yamada N, Sanada Y, Katano T, Tashiro M, Hirata Y, Okada N, Ihara Y, Miki A, Sasanuma H, Urahashi T, Sakuma Y, Mizuta K. Pediatric living donor liver transplantation for congenital hepatic fibrosis using a mother's graft with von Meyenburg complex: A case report. *World J Gastroenterol* 2016; 22(44): 9865-9870 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i44/9865.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i44.9865

INTRODUCTION

Multiple biliary hamartoma is a rare, benign lesion known as von Meyenburg complex[1]. The frequency is unknown, but Lin *et al*[2] have reported that it was seen in 0.35% of patients undergoing liver biopsy. Multiple biliary hamartoma is a benign tumor and does not affect liver function. However, there exist only two case reports of liver transplantation (LT) with a donor having von Meyenburg complex, both of which were orthotropic liver transplantation LT[3,4]. Due to the lack of information, graft liver function with von Meyenburg complex and the risk of malignancy in the graft liver are unknown.

To the best of our knowledge, there have been no reports of living donor liver transplantation (LDLT) from a donor with von Meyenburg complex. We herein report a case of pediatric LDLT for congenital hepatic fibrosis (CHF) with a graft taken from the mother having von Meyenburg complex, and considered the possibility of a liver with von Meyenburg complex for a further expansion of living donor pool.

Interestingly, the recipient (the donor's daughter) had CHF, which is considered to be associated with von Meyenburg complex. Both of these conditions are considered as type of hepatobiliary fibropolycystic disease[5]. The case especially focuses on the pre-operative imaging findings and liver function of the donor and the recipient.

CASE REPORT

A 6-year-old female patient with CHF was referred to our hospital for LT. She developed sudden hematemesis due to esophagogastric varices and had been diagnosed with CHF based on histological findings from a liver biopsy that was done when she was 4 years old. She had been suffering from recurrent gastrointestinal bleeding for 2 years and was considered competent for LT (Figure 1A, Table 1).

The only living donor candidate was the patient's 38-year-old mother. The mother had undergone clinical examinations required for evaluation as a potential donor. Her magnetic resonance imaging (MRI) scan showed multiple 3-5 mm nodules with high intensity in T2 weighted imaging (Figure 2A). The nodules were dominant in the right lobe of her liver, although they were seen in the left lobe to some extent. Her computed tomography (CT) scan also revealed multiple low-density areas that were 3-5 mm in diameter, and had higher density in CT scan than the water level (Figure 2B). Therefore, rather than multiple liver cyst, multiple biliary hamartoma, also known as von Meyenburg complex was suspected. Her standard liver function tests were normal (T-Bil; 0.33 mg/dL, AST; 12 U/L, ALT; 11 U/L), and she did not have cystic disease of any other organ, especially polycystic kidney disease. Although her daughter developed repetitive severe gastrointestinal bleeding, no other living donor was available, and therefore the mother was selected as the living donor.

During LDLT, hepatectomy of the donor's left-lateral segment (LLS) was performed. Multiple cystic lesions were observed in the mother's liver during surgery, more so in the right lobe of the liver. The LLS graft from the donor weighed 250 g, and it was estimated to be 22.7% of the donor's total liver volume of the donor. The operation time and amount bleeding were 4 h 24 min and 430 mL, respectively. The operation and post-operative course of the donor were uneventful and she was discharged on post-operative day (POD) 8.

During the recipient's surgery, the graft liver was transplanted after total hepatectomy. Her native liver showed fibrotic changes in the portal area, with proliferation of the pseudocholangiolar ducts, and it was consistent with CHF (Figure 1B-D). The graft-recipient weight ratio and graft volume/standard liver volume were 1.19% and 43.2%, respectively. The vascular reconstructions were anastomosed from the donor's left hepatic vein to the recipient's middle and left hepatic vein, the donor's left portal vein to the recipient's main portal vein, and the donor's left hepatic artery to the recipient's right hepatic artery. After reperfusion of the graft liver, we observed multiple small lesions suspected to be biliary hamartoma (Figure 3A). Time zero biopsy of the graft liver revealed the

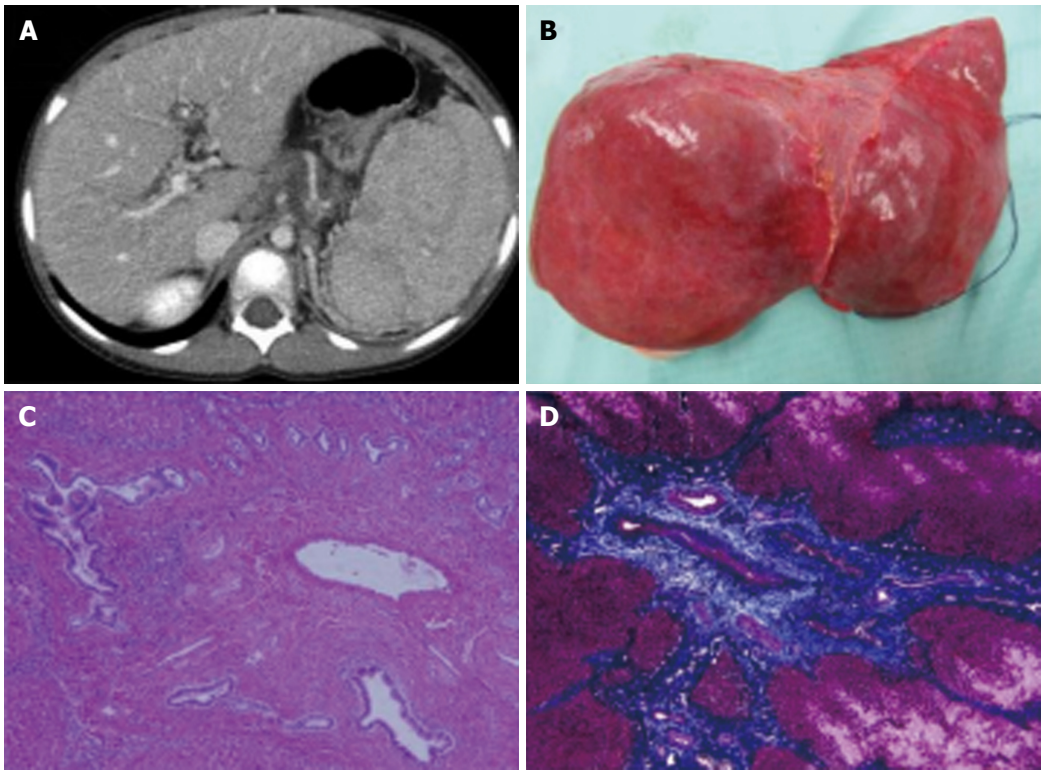

Figure 1 Computed tomography scan (A), macroscopic finding (B) and histology findings (C, D) of the recipient. A, B: Reveals splenomegaly due to portal hypertension (A), macroscopic finding of the recipient's native liver (B). Her native liver shows fibrotic changes in the portal area with proliferation of the pseudocholangiolar ducts, which is consistent with congenital hepatic fibrosis (C, D). C: Hematoxylin-eosin staining; D: Azan staining. Magnification × 100.

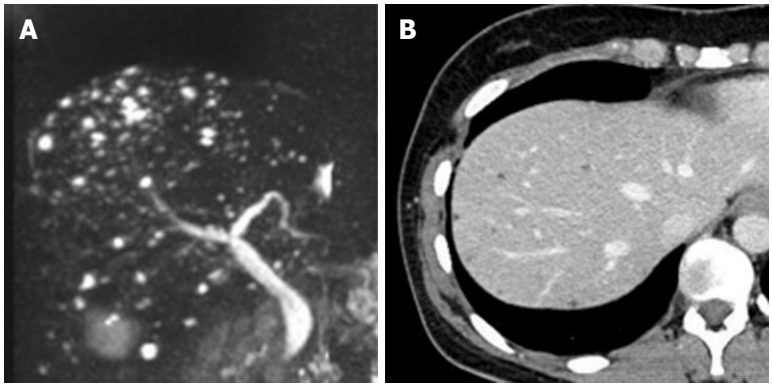

Figure 2 Magnetic resonance imaging and computed tomography scan of the donor. Magnetic resonance imaging scan of the donor shows 3-5 mm multiple nodules with high intensity in T2 weighted image (A). Her computed tomography scan also reveals multiple low-density area of 3-5 mm in diameter. Multiple biliary hamartoma, also known as von Meyenburg complex, was suspected.

slight fibrosis around portal area, with a Metavir fibrosis score F0 (Figure 3B). The operation time and estimated bleeding volume were 10 h 20 min and 380 mL, respectively. Tacrolimus and Methylprednisolone were used as postoperative immunosuppressive therapy. After LDLT, the recipient developed catheter-related bloodstream infection on POD 7 and obstruction of external biliary drainage tube on the POD 22, which was accompanied by slight temporary elevation of alanine transaminase (Figure 4). However, the graft liver function was good, and gastrointestinal bleeding did not occur after LDLT. She was therefore discharged on POD 31. Her routine CT scan on POD 28 revealed some small nodules suspected to biliary hamartoma (Figure 3C). One year have passed after LDLT, and her graft liver and renal function were normal. Her oncogenic surveillance was also with no abnormal findings.

DISCUSSION

Kerr *et al*[6] first reported in 1961 that CHF is a hereditary autosomal recessive fibropolycystic disease of the liver. It is caused by ductal plate malformations

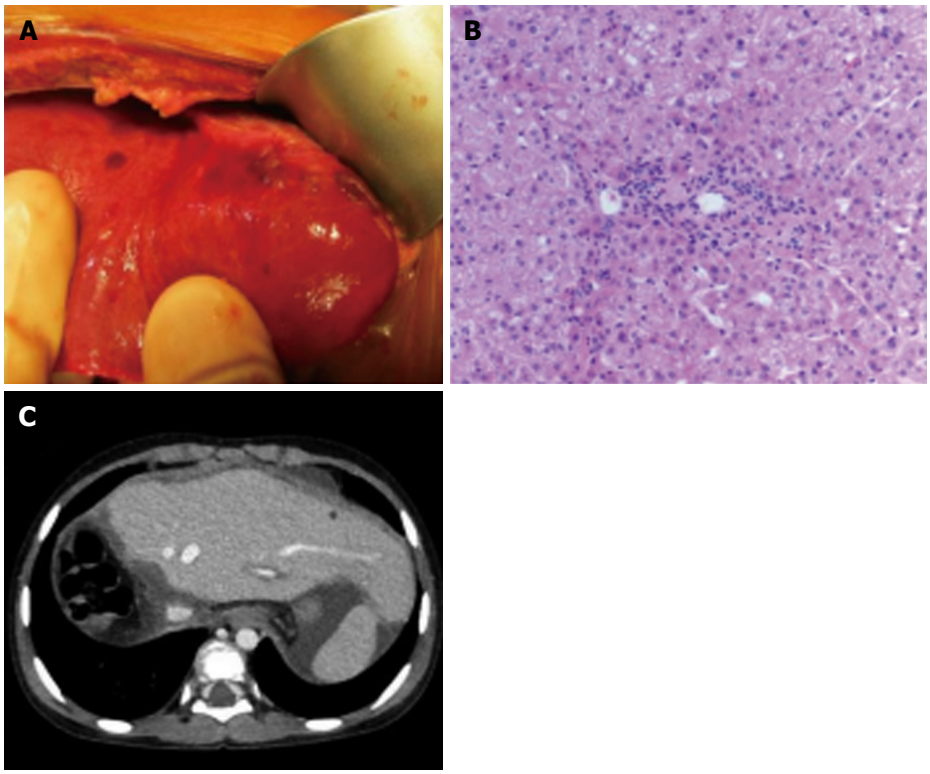

Figure 3 Clinical findings during the recipient's surgery. During living donor liver transplantation, we identified the small lesions of the graft liver suspected as biliary hamartoma after reperfusion (A). Time zero biopsy of the graft liver revealed slight fibrosis around the portal area; metavir fibrosis score F0 (B) (magnification × 100). Computed tomography scan on postoperative day 28 (C).

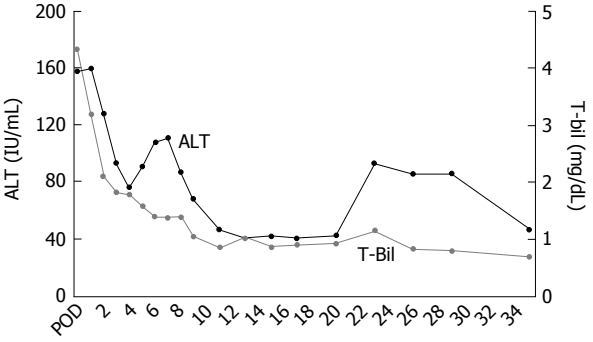

Figure 4 Clinical course of the recipient after living donor liver transplantation. Her graft liver function was good and she was discharged on postoperative day 31. ALT: Alanine transaminase; T-Bil: Total bilirubin.

Table 1 Preoperative blood examination of the recipient

Items	Results	Units
WBC	3.6×10^4	/μL
RBC	372	/μL
Hb	7.9×10^4	g/dL
Ht	26.4	%
Plt	6.9	/μL
TP	6.4	g/dL
Alb	4.2	g/dL
BUN	10	mg/dL
Cre	0.24	mg/dL
T-Bil	0.67	mg/dL
AST	43	U/L
ALT	39	U/L
LDH	258	U/L
γ-GTP	41	U/L

WBC: White blood cell count; RBC: Red blood cell count; Hb: Hemoglobin; Ht: Hematocrit; Plt: Platelet count; TP: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; Cre: Creatinine; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; γ-GTP: γ-glutamyltransferase.

and is sometimes accompanied by polycystic kidney disease[7,8]. The typical pathological findings of a liver with CHF are progressive fibrosis around the portal veins and cystic changes of the peripheral small-sized bile ducts in the thick fibrotic band. Survival depends mainly on complications of portal hypertension, such as variceal bleeding and hypersplenism, and some cases are complicated with cholangiocarcinoma or hepatocellular carcinoma in the long term. There are some patients with CHF who progress to liver failure or severe gastrointestinal bleeding, and require LT.

Hepatobiliary fibropolycystic disease, including Caroli disease, von Meyenburg complex (multiple biliary hamartoma), CHF, and polycystic liver disease are considered to arise from the same etiology, with different levels of the biliary tract being affected. von Meyenburg complex is now considered to not be a pure liver disease, but rather a multi-organ disorder involving the brain, portal vein, kidneys, and bile ducts; some genetic linked disorders have been identified[9-11].

Interestingly, this is the first case report wherein a parent has von Meyenburg complex and the child has CHF, but neither had multi-organ disorder.

Only two case reports exist of LT from patients with von Meyenburg complex; both of these cases are orthotropic LT. They were diagnosed at procurement of LT, and the recipient's graft liver function was good after LT. Our case is likely the first case report of LDLT from a donor having von Meyenburg complex. We could not obtain the histopathological diagnosis of the biliary hamartoma; however, the MRI and CT findings of von Meyenburg complex are characteristic and reportedly enable a highly accurate diagnosis[12,13]. Although some lesions of von Meyenburg complex were seen in the donor's left lobe, they were dominant in the right lobe, and preoperative laboratory liver function tests in the donor were normal. There was no other living donor available, due to which the LLS graft with some lesions of von Meyenburg complex had to be retrieved. Although time zero biopsy of the graft revealed slight fibrosis in the portal area, the graft function and clinical course of the recipient were good. To our experience and according to previous reports, graft liver with von Meyenburg complex shows no problem regarding graft function in the early post-LT phase.

The von Meyenburg complex usually consists of benign tumors, but there have been several case reports of cholangiocarcinoma or hepatocellular carcinoma arising from the liver with the multiple biliary hamartoma[14-17]. To date, the frequency of cholangiocarcinoma arising from a liver with von Meyenburg complex is unknown; whether these cholangiocarcinomas were derived from the biliary hamartoma is also unknown. However, under the circumstances, oncologic surveillance of the graft liver in the recipient should be performed over time. In addition, hepatobiliary fibropolycystic disease, including CHF, is sometimes accompanied by polycystic kidney disease, which can result in renal failure[18]. When following up the LT recipient for CHF in the outpatient clinic, physicians should check renal function as well as for the appearance of cysts in the kidney.

In our institute, we perform the routine blood test (including standard liver function test and renal function test) and echo check per 3 mo for first 5 years and per 6 mo after that. We also examine the CT scan at 1 mo, 6 mo, and 1 year after LT. From 1 year to 5 years after LT, CT scan were performed annually; thereafter they were examined every 2 years[18]. We will perform same follow up for the recipient. In addition, we will add the tumor marker test (carcinoembryonic antigen, carbohydrate antigen 19-9), when blood examination will be performed.

In conclusion, we successfully treated a patient with CHF by performing LDLT using the mother's graft that had von Meyenburg complex. Long-term follow-up regarding graft liver function, screening for malignant tumors, checking renal function, and watching for the appearance of polycystic kidney disease is needed.

COMMENTS

Case characteristics

A 6-year-old girl with congenital hepatic fibrosis (CHF), who suffered from recurrent gastrointestinal bleeding, was transplanted her mother's liver graft with von Meyenburg complex.

Clinical diagnosis

The recipient was suffered from hematemesis. The donor had no symptoms; the von Meyenburg complex was detected after donor's examination.

Differential diagnosis

Hepatobiliary fibropolycystic disease, including Caroli disease, von Meyenburg complex (multiple biliary hamartoma), CHF, and polycystic liver disease.

Laboratory diagnosis

The donor with von Meyenburg complex was normal liver function.

Imaging diagnosis

The donor's magnetic resonance imaging scan showed multiple 3-5 mm nodules with high intensity in T2 weighted imaging.

Pathological diagnosis

The recipient was diagnosed CHF.

Treatment

Living donor liver transplantation (LDLT).

Related reports

There exist only two case reports of liver transplantation with a donor having von Meyenburg complex, both of which were orthotropic liver transplantation.

Term explanation

Multiple biliary hamartoma is a rare, benign lesion known as von Meyenburg complex, which is included in the hepatobiliary fibropolycystic disease.

Experiences and lessons

LDLT using a graft with von Meyenburg complex is safe and useful for a further expansion of living donor pool. Long-term follow-up is needed with respect to graft liver function and screening malignant tumors.

Peer-review

This paper is informative and is suitable for publishing.

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