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Genome-based nutrition: An intervention strategy for the prevention and treatment of obesity and nonalcoholic steatohepatitis

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location. In Latin America, most countries, including Mexico, have a heterogeneous admixture genome with Amerindian, European and African ancestries. However, certain high allelic frequencies of several nutrient-related polymorphisms may have been achieved by past gene-nutrient interactions. Such interactions may have promoted the positive selection of variants adapted to regional food sources. At present, the unbalanced diet composition of the Mexicans has led the country to a 70% prevalence rate of overweightness and obesity due to substantial changes in food habits, among other factors. International guidelines and intervention strategies may not be adequate for all populations worldwide because they do not consider disparities in genetic and environmental factors, and thus there is a need for differential prevention and management strategies. Here, we provide the rationale for an intervention strategy for the prevention and management of obesity-related diseases such as non-alcoholic steatohepatitis based on a regionalized genome-based diet. The components required to design such a diet should focus on the specific ancestry of each population around the world and the convenience of consuming traditional ethnic food.

Key words: Latin America; Mexico; Gene-nutrient interactions; Evolution; Food history; Western diet; Nonalcoholic steatohepatitis; Obesity

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Core tip: New intervention strategies for the prevention and management of obesity and associated gastrointestinal diseases are warranted due to their chronic complications. In the era of genomic medicine and nutritional genomics, we are now closer to understanding how unbalanced gene-nutrient interactions are involved in the onset and progression

Abstract

Obesity and nonalcoholic steatohepatitis are increasing in westernized countries, regardless of their geographic

of these diseases. The implementation of regionalized diets based on the genetic ancestry and natural staple food sources of each population may result in better health and nutrition worldwide. Further studies are required to tailor the appropriate diet for each type of population to win the battle against obesity and associated co-morbidities.

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INTRODUCTION

Overweightness and obesity have been relatively accepted as the conspicuous culprit associated with the increasing incidence of metabolic-related co-morbidities^[1,2]. The rate by which the prevalence of obesity has increased in the last decades has led health experts to estimate that 1.4 billion adults are overweight globally, and of these overweight adults, 300 million are obese^[3]. In addition, obesity has grown markedly faster among the developing countries in a shorter time span than the developed world^[4]. In consequence, regardless of whether populations are geographically located in the Eastern or Western hemisphere, populations that have rapidly adopted a westernized lifestyle are now immersed in an obesogenic environment^[5]. This environment is characterized by the consumption of calorie-dense foods, reduced physical activity, and greater psychosocial stress driven by macro-level factors of globalization^[6,7]. Unfortunately, as the world's adult population obesity increases, the next human generations are becoming more susceptible to gaining weight at earlier stages of life. Estimations using World Health Organization data have shown that global childhood obesity increased from 4.2% in 1990 to 6.7% in 2010^[8]. Furthermore, as this trend continues to rise, the relative risk of morbidity and mortality due to premature type 2 diabetes mellitus (T2DM)^[9] and cardiovascular disease (CVD)^[10] is rising accordingly.

The link between obesity and the increasing prevalence of associated chronic illness is that obesity is more than an input-output energy ratio imbalance^[11]. Obesity generates a highly complex multisystem deregulation of the glucose and lipoprotein metabolism orchestrated by insulin resistance invoked through an excess of serum fatty acids^[11]. Insulin resistance is a key component of the metabolic syndrome that ultimately leads to cellular oxidative stress and low-grade systemic inflammation affecting several tissues and organs^[12]. One such organ is the liver. Thus,

the next-in-line co-morbidity after viral hepatitis and alcoholic liver disease may be non alcoholic fatty liver disease (NAFLD), which comprises fatty liver and nonalcoholic steatohepatitis (NASH)^[13,14]. Unchecked, these conditions may lead to fibrosis/cirrhosis and hepatocellular carcinoma^[15-17]. However, despite the similarity in the rising worldwide pattern of obesity, the myriad causal-effect relationships involved in the pathogenesis of NAFLD/NASH are not fully understood^[18]. Moreover, virtually all stages of progression, from obesity to long-term complications, may be modulated by hereditary and environmental factors^[11,18]. Hence, the wide variety of abnormal metabolic phenotypes derived from the obese state may be due to disparities in the population's distribution of gene polymorphisms interacting with nutritional factors.

Currently, genomic sciences are providing us with a better understanding of how nutrients interact with the human genome and the impact of natural selection on genes involved in modern-day complex diseases^[19]. Additionally, variations in the allelic frequencies of nutrient-related polymorphisms may mark the differences in risk of complex diseases among populations^[20]. Moreover, human societies that have conserved their staple food diet are less prone to nutrition-related diseases^[4,21]. Thus, prevention and treatment strategies for obesity-related diseases should be based on the rationale of a regionalized genome-based diet rather than a one-size-fits-all approach^[5]. The components of such a diet should focus on the genetic susceptibility and the traditional food culture of each population. Thus, the aim of this editorial is to describe several gene-diet interactions that may contribute to obesity and NAFLD/NASH. We conclude with a genome-based nutrition intervention strategy that defines the best dietary resources according to the individual's background.

EVOLVING HUMAN GENOME-NUTRIENT INTERACTIONS

Evolutionary genomics has offered insights on how new climates, diet, and infectious diseases exert positive selective pressures on the human genome, especially within human subpopulations^[20,22]. Hunting the genome for "signatures" of positive selection has led scientists to parts of metabolic gene sequences that evolve more rapidly than others when exposed to environmental challenges^[20,23,24]. However, these adaptive challenges can occur in distinct geographic areas, rendering differences in the frequency of alleles of the single nucleotide polymorphisms (SNPs) that allow carriers to adapt to such environmental challenge^[20,25,26]. Interestingly, this dynamic interaction between genes and diet also seems to be mediated by culture practice. For example, a recent discovery was the finding that the marine microbe *Zobellia*

Table 1 Allelic frequencies of nutrient-interacting genes in America and worldwide

Gene	Allele	Population	Frequency (%)	Ref.
MTHFR	677T	Huicholes (Native Mexican)	56.0	Dávalos <i>et al</i> ^[28] 2000
		Asian	36.7	HapMap-JPT ^[29]
		European	23.7	HapMap-CEU ^[29]
		African	11	HapMap-YRI ^[29]
TAS2R38	AVI	European	47	Kim <i>et al</i> ^[30] 2003
		Asian	30	
	PAV	Southwest Native American	100	
		Asian	70	
AMY1	Copy number	European	49	
		European-American (high starch diet)	6.8 ¹	Perry <i>et al</i> ^[31] 2007
		Mexican (high starch diet)	6.1 ¹	Mejía-Benítez <i>et al</i> ^[32] 2014
		Biaka African (low starch diet)	5.47 ¹	Perry <i>et al</i> ^[31] 2007
CD36	-31118A	Yakut Asian (low starch diet)	5.24 ¹	
		Egyptian	67.5	Bayoumy <i>et al</i> ^[33] 2012
		Caucasian	53.6	Ma <i>et al</i> ^[34] 2004
		African American	43.8	Keller <i>et al</i> ^[35] 2012
ABCA1	230C	North Indian	38.2	Banerjee <i>et al</i> ^[36] 2010
		Xavantes (Native Brazilian)	31	Acuña-Alonzo <i>et al</i> ^[37] 2010
		Coras (Native Mexican)	29	
		European	0	
APOE	E2	Asian	0	
		African	0	
		African	19	Singh <i>et al</i> ^[38] 2006
		European	12.7	
	E4	Asian	4.6	
		Huicholes (Native Mexican)	0	
		Huicholes (Native Mexican)	28.7	Aceves <i>et al</i> ^[39] 2006
		African	27	Singh <i>et al</i> ^[38] 2006
LCT	-13910T	Asian	10.5	
		European	1.1	
		European	39.1	Corella <i>et al</i> ^[40] 2010
		White Brazilian	24.7	Mattar <i>et al</i> ^[41] 2009
		Mapuches (Native Chilean)	22.2	Morales <i>et al</i> ^[42] 2011
		Black Brazilian	12	Mattar <i>et al</i> ^[41] 2009

¹Mean copy number.

galactanivorans may have transferred algae-digesting enzymes to the human gut bacterium *Bacteroides plebeius*^[26]. This microbe contains a B-prophyranase gene similar to one identified in the marine bacterium that breaks down algae carbohydrates, as in the food nori, which otherwise would be indigestible. However, to date, only people of Japanese ancestry, who have a legendary consumption of nori-made sushi-rolls and other algae-based foods, are gifted with this type of microbiota. Another example is the lactase persistence trait: the ability to digest fresh milk and other dairy products into adulthood is more frequent in pastoralist and dairying populations of northern Europeans and in certain African and Arabic nomadic groups, in contrast to the rest of the world^[27]. Likewise, among the Latin American countries, milk was never a genetically recognized food among the Amerindians until the arrival of the Europeans.

In Table 1, several nutrient-interacting genes are depicted to illustrate their contrasting allelic frequencies worldwide, including the Americas. The methylenetetrahydrofolate reductase (*MTHFR*) enzyme involved in the one-carbon metabolism^[28,29], the taste receptor 2R38 (*TAS2R38*) for the perception of bitter

and pungent substances^[30], amylase 1 (*AMY1*) to digest complex carbohydrates^[31,32], lipid metabolism genes: Class B scavenger receptor (*CD36*)^[33-36], ATP binding cassette transporter (*ABCA1*)^[37] and Apolipoprotein E (*Apo E*)^[38,39], and lactase (*LCT*) enzyme^[40-42] all express population-based allele dominance that may define differential dietary requirements within humans^[20,35,36]. Moreover, these adaptive genes that were once shaped in a specific natural environment may now become disease alleles due to the rapid shifting man-made surroundings or even recent genetic admixture of a given population^[43-45]. Therefore, in the following section, we explain the genetic basis and food history common to the American population of which Mexico is representative.

AMERINDIAN ANCESTRY AND FOOD HISTORY IN LATIN AMERICA

Early years: First settlers and native food sources

The indigenous Americans descend from at least three streams of gene flow, and archaeological evidence shows that early settlers in Mexico date back to 30000

years ago^[46,47]. The nomadic lifestyle of the initial ancestors and the climatic changes conditioned their southward expansion through the American continent^[48]. The initiation of the food history in septentrional Latin America begins in two pre-Hispanic geographical regions with distinct ecosystems. Aridoamerica, an extraordinarily biodiverse dryland situated in the north and central region, was the home of small and isolated semi-nomadic groups living a Paleolithic lifestyle^[49]. In contrast, Mesoamerica was a territory that extended from the middle region of Mexico to the northern part of Central America. It has incredible natural biodiversity, especially in the Mexican Basin, which has since early times drawn nomadic groups of hunter-gatherers to becoming sedentary societies eventually^[50,51]. They were small groups of people living on a Paleolithic diet consisting of wild plants, lacustrine animals, and hunting small animals, followed by big game^[50]. The adequate climatic conditions and environment of Mesoamerica allowed the first cultivation of plants (5500 BC). Finally came the emergence of agriculture and the development of the Neolithic sedentary societies (2500 BC; Pre-Classic stage)^[51].

The development of several agricultural societies was the starting point of a new food chain system that allowed the consumption of a mixed diet based on cultivated plants such as maize, squash, chili, avocado, edible green leafy vegetables known as "*quelites*", amaranth, chia and beans^[45,50,51]. However, it also included turtle meat, deer, domesticated dogs and other foods obtained by fishing, hunting and gathering practice. In the following years, comprising the Classic (150-900 AC) and Post-Classic (900-1519 AC) stages, the pre-Hispanic cultures developed, grew and spread along with intensive agricultural production using the *milpa* (cornfield combined with other staple plants) and *chinampas* systems (wetland agriculture)^[50,51]. Before the conquest, the most developed population was *Tenochtitlan* (the Aztec capital city). By this time, the food regime of most all the neighboring ethnic groups was mainly the pre-Hispanic diet that will be discussed in section IV. Meat was uncommon for most people, and its consumption was reserved for the "nobles" or at special ceremonies; instead, most of the population ate several species of worms, insects, and wild herbs that were a rich source of protein. These ancestors took wisely what was given by nature and turned it into peculiar tasty dishes. Furthermore, they discovered the healing powers of food, what to avoid and eat to prevent and cure diseases.

Conquest and colonial times: The initial genetic and food culture admixture

In 1519, the Spaniards arrived. The genetic and cultural admixture of the Amerindian forefathers began with the European colonization that continued from the conquest of *Tenochtitlan* in 1521 until

1851^[52]. The Spaniards introduced a wide variety of crops and domestic animals that allowed them to continue their own food habits. Foodstuffs such as wheat, sugar cane, cattle, pigs, sheep, goats, chicken, radish, lettuce, cabbage, cucumber, pomegranate, pear, apple, grape, fig, peach, and oils, among others were brought^[45]. Thus, the original diversity of the rich pre-Hispanic sources of nutrients was diminished due to eradication by the Spaniards of all food that was related to non-Christian religious ceremonies or unfamiliar to their taste buds. They abandoned some foods such as amaranth and chia, rich in proteins and polyunsaturated fats, yet on the other hand, a new admixture of novohispanic dishes arose.

Over time, the Amerindian population decreased due to warfare, overwork and the presence of epidemic diseases, allowing the widespread settlement of Europeans together with the almost complete imposition of their culture, followed by the arrival of slaves from several regions of Africa^[52]. These three populations were the founder races that originated the genetic admixture of the early mestizos, socially known as "*las castas*", which prevailed during the 300 year Colonial period^[45]. This time served as the cradle of the genetic and cultural differences that continue in present-day Mexico, which also occurred among other Latin American countries.

Gradual transformation of food habits

Intertwined with the early historical events of Mexico's Independence (1821) and Revolution (1921) came the gradual industrial growth from the 17th through the 18th century that brought new foreigners to Mexico. In recent years, immigration has shaped the present-day gene pool of the Mexican population^[53]. Thus, genome-wide analysis has shown that the genetic architecture of the Mexican population and of most Latin American populations is a heterogeneous admixture of Amerindian, European and African ancestries^[39,46,48]. However, the percentage of each ancestral component varies with region, contributing to the overall heterogeneity^[39,53,54].

Mexico's food history provides an excellent setting to explore the effect caused by the interaction between ancestral genes and the native food regimen, one that might have exerted selective pressures on certain SNPs related to food metabolism. Having been positively selected, they served for survival in the ancestral environment; however, at present, they may have become detrimental. In the last five hundred years, the Mexican population has "progressed" from a society with a traditional lifestyle to a modern lifestyle along with an unfortunate nutrition transition. Thus, in the following section, we describe some examples of mismatched gene-nutrient interactions and their plausible association with metabolic liver disease.

GENETIC ADAPTATIONS FOR REGIONAL FOOD SOURCES

Vegetables

MTHFR C677T polymorphism: The Amerindian's pre-Hispanic diet was rich in a wide variety of vegetables that provided the vitamins and minerals needed to prevent nutritional deficiencies. Many indigenous foods such as maize, green beans, avocado, chia and "*quelites*" are natural sources of folates^[45]. An extensively studied SNP is the 677T allele of the *MTHFR* gene that encodes a thermolabile enzyme with decreased activity. This enzyme catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the most abundant form of folate in the plasma^[55] and a co-substrate for homocysteine remethylation to methionine. In combination with an insufficient folate intake, it has currently been associated with neural tube defects^[56], CVD^[57,58], hyperhomocysteinemia, liver steatosis and NASH^[59-61]. However, the abundance of folates in the Amerindian's pre-Hispanic diet could have acted as a positive selection pressure for this SNP without causing any disease in the population. Evidence of genetic selection for the T allele related to folate intake has been reported^[62-64]. In regard to the Mexican population, the highest frequencies of the T/T genotype have been found among native groups with a high Amerindian ancestry compared with other world populations, as shown in Table 1.

TAS2R38 haplotypes: The ability to taste bitter substances such as the ones found in cruciferous vegetables as well as the perception of sweet taste, the pungency of chili peppers and the texture of fats varies within human populations. In this connection, studies have suggested that exposure to these food substances may have been an important factor in the evolution of this trait^[65]. This ability is sustained by the genetic variability of three functional SNPs in the *TAS2R38* gene^[66] that have led to the existence of two amino acid haplotypes: alanine, valine, isoleucine (AVI), and phenylalanine, alanine and valine (PAV). AVI/AVI homozygotes present the lower bitter taste sensitivity (non-tasters), whereas PAV/PAV homozygotes show the highest sensitivity to these flavors (tasters)^[66,67] (Table 1). Consistently, it has been reported that AVI/AVI homozygotes consume more bitter cruciferous vegetables than either PAV/AVI heterozygotes or PAV/PAV homozygotes^[68]. Therefore, being a non-taster may have significant health benefits, as bitter tasting foods such as grapefruits, coffee and cruciferous vegetables have been recognized for their antioxidant properties.

In regard to the pre-Hispanic diet, the wide variety of chili plants (*Capsicum* spp.) of Mesoamerica were essential ingredients of the staple diet and thus a good source of vitamins such as A and C^[45]. However, the

tolerance for both the pungency of capsicum, the main "hot" component of the chili plant, and for the bitter taste of the *quelites* may have required the presence of a non-taster phenotype. Thus, the non-tasting for *quelites* allows on the one hand the acquisition of adequate amounts of dietary folates, which, in conjunction with the aforementioned *MTHFR* C677T SNP, allows a proper metabolism of homocysteine and the final endogenous production of glutathione. Currently, vitamins A and C have been studied for their antioxidant properties in the treatment of liver diseases, although glutathione is a clinically significant antioxidant because low levels play an important role in the pathogenesis of NAFLD^[64].

Legumes and cereals

Copy number of *AMY1* gene: Our Amerindian predecessors were creators of complex agricultural systems, nearly 7000 years ago. In the *milpa* and the *chinampas* grew many new foods, some of which contained a high content of starch, such as maize and beans^[45]. Therefore, as in other agricultural societies, it may be inferred that the Mexican population is genetically adapted to diets high in complex carbohydrates. This dietary change increased the need for a higher protein levels of salivary amylase (enzyme responsible for starch hydrolysis), which has been associated with an increase in the number of copies of the gene encoding it (*AMY1*)^[69]. It has been hypothesized that natural selection may have influenced the variation of the *AMY1* copy number in human populations with traditionally high-starch diets, thus improving the efficiency by which these foods are digested in the gastrointestinal tract^[31]. Some studies have shown that *AMY1* copy number is positively correlated with the level of amylase protein expression in saliva^[68,69]. Furthermore, it was found that the mean diploid *AMY1* copy number is higher in individuals from agricultural populations with diets rich in complex carbohydrates (European-Americans and Japanese) than individuals from populations with diets including relatively few starchy foods (Datog, Mbuti and Biaka in Central-East region of Africa and the Yakut in Asia) (Table 1). *AMY1* copy number has been recently studied in the Mexican population, in which a high copy number of the *AMY1* gene may protect against obesity^[32]. However, a high intake of simple carbohydrates in the diet has showed correlation with obesity and severity of fatty liver in the absence of traditional risk factors^[70,71].

Fats and cholesterol

CD36 gene: The overconsumption of high-fat foods depends upon their high palatability and taste perception^[72-75]. Class B scavenger CD36 receptor plays a fundamental role in the taste perception of dietary fat^[76] by capturing long-chain fatty acids into the cell^[77]. Thus, the genetic variability of the *CD36*

gene could explain the differences in fat perception and fat preferences across individuals^[35] (Table 1). It has been reported that SNP -31118G>A in the promoter region predicts the oral responses and preference for dietary fat in adults of African-American ancestry by reducing the CD36 expression^[78,79]. Positive selective pressure may have favored the A allele in the native Amerindians because the composition of their habitual diet has been low in fat, thus maintaining low levels of the CD36 receptor. However, exposure to the obesogenic environment in which the country is currently immersed could favor the consumption of high-fat foods and obesity.

ABCA1 R230C polymorphism: Foods such as avocado, squash seeds, cacao, and chia, as well as lacustrine resources and the lean meat of certain animal species were the staple fat sources from our ancestors' diet^[45]. These sources were characterized primarily by providing polyunsaturated fatty acids and low amounts of saturated fat and cholesterol^[37]. ABCA1 is the major transmembrane transporter that mediates the efflux of cholesterol and phospholipids from cells to apolipoprotein A- I (apoA- I) to generate nascent HDL particles^[80]. Thus, the liver not only participates in synthesizing these nascent HDL particles, which are transported to the periphery for reverse cholesterol transport, but also serves as a source of cholesterol for plasma HDL acceptors (including ovary, adrenal and testis tissues). Therefore, the liver and peripheral cells modulate the intracellular level of cholesterol by the level of expression of the ABCA1 transporter^[81].

However, the non-synonymous variant R230C of the ABCA1 gene has been associated with low HDL cholesterol levels because it reduces the cholesterol efflux by 27%. Interestingly, this variant has shown evidence of recent positive selection in Native-Americans, given that it has been found to be exclusive to Native American and Native American-derived populations. It has been speculated that 230C carriers could have had a selective advantage, due to a lower cholesterol efflux, that could favor the storage of intracellular cholesterol and energy to survive periods of famine and adapt to low-fat diets. However, under current westernized lifestyle changes, the 230C allele may represent a disadvantage for low HDL cholesterol levels (hypoalphalipoproteinemia), indeed one of the most common dyslipidemia in Mexicans^[37]. Moreover, this variant has also been associated with higher body mass index and NAFLD^[82].

Apo E polymorphism: The Apo E gene encodes a plasma glycoprotein that is part of the structure of triglyceride-rich lipoprotein (VLDL, HDL, chylomicrons). Thus, Apo E protein mediates their metabolism in the liver and acts as a ligand for low-density lipoprotein (LDL) receptors. Three alleles (E2, E3, and E4) determine six genotypes with well-described amino acid substitutions at positions 112 and 158^[83]. Such

substitutions confer differential binding affinities for their respective receptors. Apo E3 allele is the most frequent isoform that allows the proper binding of Apo E-containing lipoproteins to their receptors (E/B, rLDL)^[83]. However, the E2 isoform binds defectively to the LDL receptors, whereas the E4 isoform has a higher affinity for triglyceride-rich lipoproteins that increases the liver uptake of these lipoproteins; consequently, LDL receptors are down-regulated^[83,84]. Apo E4/E4, E4/E2 or E4/E3 carriers tend to have higher serum levels of LDL and total cholesterol, compared with their E2 allele counterparts^[85]. However, the E2 allele confers genetic susceptibility to hypertriglyceridemia. In West Mexico, this allele has been associated with hypertriglyceridemia and early onset of alcoholic cirrhosis^[86].

The distribution of the Apo E alleles varies both globally and within the admixture Mexican population (Table 1). This genetic variation has been linked with differences in the prevalence and predominance of dyslipidemia reported among the population, as well as their interaction with environmental factors, such as diet. Although the Apo E2 allele has been associated with European ancestry, Apo E3 is predominant among the inhabitants of Central Mexico, and the Apo E4 allele has been associated with African ancestry or Amerindian groups^[46]. To date, this allele has one of the highest rates worldwide within the Huicholes population from West Mexico (Table 1). As the E4 allele reduces the efficiency of cholesterol metabolism, these native carriers could have been protected by their low-fat diet in their natural environment, reinforced by the ABCA1 polymorphism. However, it may become a risk allele when these carriers consume a high-fat urban diet.

Milk and dairy products

Lactase: Lactase is an enzyme expressed in the intestinal microvilli, which hydrolyzes the disaccharide lactose made up by glucose and galactose. In newborns, this enzyme is highly expressed to digest human milk. After weaning, a typical phenomenon known as "lactase non-persistence" takes place and is characterized by the decreased enzyme expression. As a result, the adult lactase activity declines, and lactose cannot be hydrolyzed, presenting poor absorption. However, this result commonly occurs in the presence of C-13910T allelic polymorphism at the promoter region of the lactase gene LCT. Among the Europeans, the -13910T allele has been associated with lactase persistence in adulthood, with a prevalence of this phenotype reaching 90%^[87]. In this case, positive selection of this polymorphism, approximately 5000 years ago, could be related to the long history of cattle domestication and consumption of dairy products in this population^[88]. In contrast, cattle and dairy products were absent in the Amerindian's pre-Hispanic diet because they were introduced quite recently, after the arrival of the Spaniards^[45]. Although

Table 2 Hepatopatogenic diet of the general population of West Mexico (*n* = 425)

Nutrient	Dietary reference values ¹	mean \pm SD
Protein	15%	17.3 \pm 4.2
Total fat	< 30%	35.5 \pm 8.3
SFAs	< 7%	10.0 \pm 3.8
MUFAs	20%	11.5 \pm 4.8
PUFAs	10%	4.6 \pm 2.5
Cholesterol (mg)	< 200	254.3 \pm 144
Total carbohydrates	50%-60%	48.6 \pm 8.8
Simple carbohydrates	< 10%	16.7 \pm 12.5
Fiber (gr)	30	15.5 \pm 9.2
Vitamin A (μ g)	1000	805.1 \pm 679
Vitamin C (mg)	60	86.4 \pm 97.9
Folic acid (μ g)	200	148.7 \pm 107
Vitamin E (mg)	10	2.8 \pm 4.3
Iron (mg)	15	13.1 \pm 6.6
Magnesium (mg)	350	219.2 \pm 123.5
Sodium (mg)	< 2400	1924.7 \pm 947
Selenium (μ g)	55-70	35.5 \pm 22.1
Zinc (mg)	15	5.7 \pm 2.8

Adapted from Ramos-López *et al.*^[95] 2013. Dietary Reference Values: References^[97,98] SFAs: Saturated fatty acids; MUFAs: Monounsaturated fatty acids; PUFAs: Polyunsaturated fatty acids.

the *C-13910* allele distribution has not yet been fully studied among the Latin American population, it is known that the lactose intolerance phenotype occurs in up to 80% of the Mexican adults^[89,90]. However, the genetic admixture, following the arrival of the Spaniards and the introduction of livestock and dairy products, has allowed certain part of the population to digest milk in adulthood. The high frequency of the lactase non-persistence phenotype indicates that humans are genetically predisposed to discontinue enzyme production because by nature, breastfeeding is essential only during the first years of life, just as cow's milk is necessary only for her calf. Moreover, given the prevalence of the *ABCA1* and *Apo E* polymorphisms in the Mexican population, dairy foods are high in saturated fat and cholesterol and should be recommended with caution in individuals who have these variants. Thus, regarding the gene-environment balance, people with lactase persistence may benefit from dairy products, yet may be at risk for obesity-related diseases. Moreover, people with lactose intolerance should read the message of their genome: avoid dairy products.

Modern-day diet composition

These few examples show that the current trend of globalized (westernized) diets may not be beneficial for everyone, and increased obesity may be associated with modifications in peoples' traditional food. Moreover, not all populations worldwide are at the same stage of epidemiological transition, including nutrition transition. In contrast to Europe and the United States, it was not until the second half of the XX century that the westernized lifestyle reached the populations of Latin America^[91]. Although this region

shares geographic and ethnic/linguistic similarities, it also has considerable genetic and cultural diversity between and within countries^[92]. In consequence, the epidemiological transition has been more heterogeneous than in other regions of the world. For instance, countries such as Argentina and Chile exhibit a predominant Caucasian ancestry with consumption of a more western-type diet and have higher rates of excess weight (> 60%), whereas Central America displays a more Amerindian dietary culture, with high intake of grains and vegetables, and prevalence rates range from 30% to 55%^[92,93].

Likewise, Mexico is among the most westernized countries of the Americas and is currently in the midst of the epidemic of obesity, with an accumulated prevalence rate of about 70% among the adult population (overweightness and obesity) and 26.2% for children, which constitutes a major risk factor for T2DM, CVD and NAFLD/NASH^[93]. The National Nutrition Survey showed that the national overweight prevalence (BMI \geq 25) for adults increased significantly from 61.8% in 2000 to 71.3% in 2012^[93]. However, the more developed industrial States in Northern Mexico have very similar epidemiological indicators to the ones observed in developed countries, whereas the less developed Central and Southern Mexican States exhibit pre-transitional conditions^[94]. These disparities may be associated with the regional genetic and culture differences that have been mentioned before.

Unfortunately, our modern-day diet has shifted away from many of the healthy traditional pre-Hispanic dietary ingredients of the past. The current diet of the Mexican population is characterized by an excessive consumption of industrially sweetened beverages (high-fructose corn syrup), over-fried foods cooked in oil or lard, red meat, and confectionary foods^[95,96]. These dietary trends have changed the nutritional composition of the diet by increasing the proportional amount of saturated fatty acids and (SFAs) simple carbohydrates (SC), and have decreased the intake of fiber and important micronutrients such as vitamins and minerals. In Table 2, a representative hepatopathogenic diet of West Mexico shows that the population of this region has an excessive amount of macronutrient calories and an imbalanced intake of micronutrients with antioxidant, anti-inflammatory and anti-fibrogenic properties^[95,97,98]. It has been documented that the long-term consumption of this unbalanced diet is an important risk factor for the development of obesity and NAFLD/NASH in many countries worldwide^[6,7].

REGIONALIZED INTERVENTION STRATEGY

In 2010, the World Health Organization declared that after viral hepatitis and alcoholic liver disease, both NAFLD and NASH would be major global health

Table 3 Features of nutritional treatment for nonalcoholic steatohepatitis recommended by international guidelines

International guide	Body weight reduction	Caloric reduction	Carbohydrates	Fat	Vitamin E	Ref.
WGO	5%-10%	25%	↓ Fructose	↓ SFAs ↑ ω3:ω6 ratio	NI	LaBrecque <i>et al</i> ^[99] 2014
AASLD, ACG, AGA	3%-10%	NE	NE	NE	800 IU/d	Chalasani <i>et al</i> ^[104] 2012
AISF	0.5 kg/wk	NE	↓ Fructose	↓ SFAs	NI	Loria <i>et al</i> ^[105] 2010
ENDO CHINA	3%-10%	500-1000	NE	NE	800 IU/d	Gao <i>et al</i> ^[106] 2013

WGO: World Gastroenterology Organization; AASLD: American Association for the Study of Liver Diseases; ACG: American College of Gastroenterology; AGA: American Gastroenterological Association; AISF: Italian Association for the Study of the Liver; ENDO CHINA: Chinese Society of Endocrinology; SFAs: Saturated Fatty Acids; NE: Not specified; NI: Not indicated.

Table 4 Potential effects of dietary nutrients in the prevention and treatment of obesity and nonalcoholic steatohepatitis

Nutrient	Potential effects	Ref.
Macronutrients		
Complex CHO/DF	Microbiota modulation, protection of gut colonization by pathogenic species, reduction of energy intake	Mann <i>et al</i> ^[107] 2007
MUFAs	Increased fatty acid oxidation and inhibition of lipogenesis	Assy <i>et al</i> ^[108] 2009; Soriguer <i>et al</i> ^[109] 2006
PUFAs	Increased fatty acid oxidation and insulin sensitivity in target tissues, inhibition of lipogenesis and anti-inflammatory	Teran-Garcia <i>et al</i> ^[110] 2007; Stienstra <i>et al</i> ^[111] 2007
Micronutrients		
Vitamins C/E	Antioxidant and anti-fibrogenic	Chang <i>et al</i> ^[112] 2006; Parola <i>et al</i> ^[113] 1992
Choline/folic acid	Hyperhomocysteinemia prevention and lipid transport	Vance ^[114] 2008
Magnesium	Immunomodulatory, antioxidant and regulation of blood glucose levels	Takemoto <i>et al</i> ^[115] 2013
Vitamin D	Increased insulin sensitivity in target tissues	Takiishi <i>et al</i> ^[116] 2010
Food functional components		
Lycopene	Antioxidant, induction of detoxifying enzymes, anti-inflammatory	Ip <i>et al</i> ^[117] 2013
Polyphenols	Antioxidant, chemopreventive, immunomodulatory, apoptosis and detoxifying enzymes induction, anti-inflammatory and anti-proliferative actions	Scalbert <i>et al</i> ^[118] 2005; Fraga ^[119] 2007; Pandey <i>et al</i> ^[120] 2009
Probiotics (<i>Lactobacillus</i>)	Microbiota modulation, immunomodulatory, production of antibacterial substances and anti-inflammatory effect	Iacono <i>et al</i> ^[121] 2011

CHO: Carbohydrates; DF: Dietary fiber; MUFAs: Monounsaturated fatty acids; PUFAs: Polyunsaturated fatty acids.

problems in the upcoming years^[99]. Thus, diagnostic, therapeutic, and management options to address these illnesses should be a top priority at all healthcare levels. Several actions have been implemented to prevent or treat obesity. In general, they often pursue weight loss through lifestyle modifications, such as reducing dietary energy intake, increasing physical activity, and addressing risk behaviors in addition to pharmacological therapy or bariatric surgery^[100]. Additionally, a wide variety of commercial diets has been promoted to the general public^[101], and government agencies have acted through national campaigns, using “My Plate” from the Dietary Guidelines for Americans 2010^[102] in the United States and “El Plato del Buen Comer” from the Mexican Official Norm (NOM-043-SSA2-2012)^[103]. Regarding the management of NAFLD/NASH, most of the intervention strategies aim to treat liver disease in conjunction with the associated co-morbidities such as obesity, hyperlipidemia, insulin resistance and T2DM^[104]. These guidelines have been developed based on systematic reviews and meta-analysis studies that provide general recommendations concerning quantitative and qualitative modifications in carbohydrates, fats (SFAs and Omega 6/Omega-3 ratio) and Vitamin E^[99,105,106],

as shown in Table 3. However, the pathophysiology of obesity and NASH is highly complicated because more than one nutritional component and metabolic pathway may be affected^[95]. Several studies show that multiple nutrients other than the aforementioned may abolish the metabolic risk factors involved in obesity/NASH. These factors include dietary modifications in the macronutrient^[107-110]/micronutrient^[111-115] composition and functional components^[116-120], which have anti-inflammatory, anti-fibrotic, anti-proliferative, antioxidant and immunomodulatory functions, as shown in Table 4. Other micronutrients besides vitamin E, such as vitamins D and C, have also been suggested^[112,116]. Lycopene and polyphenols, which may be provided by distinct food sources worldwide, have pleiotropic properties^[118,120]. Furthermore, the role of probiotics^[121] in the pathogenesis of inflammatory liver disease is an ongoing topic^[122], in which prebiotics from foods are an inherent counterpart. Overall, it is obvious that many beneficial nutrients that may aid against obesity/NASH can in fact be part of a natural (non-processed) diet, one that resembles the staple ethnic diets of several traditional societies worldwide, such as the Mediterranean, Japanese/Chinese, Greek, or even Mexico and other Latin American countries. However, it

Table 5 Beneficial nutrient content of the staple diet of Mexico

Scientific name	Common name	Nutrient
<i>Salvia hispanica</i>	Chia	MUFAs, PUFAs, magnesium
<i>Theobroma cacao</i>	Cocoa	MUFAs, magnesium, polyphenols
<i>Zea mays</i>	Maize	Magnesium, choline, vitamin E, MUFAs
<i>Prunus dulcis</i>	Almond	Vitamin E
<i>Phaseolus vulgaris</i>	Bean	Magnesium, choline, PUFAs
<i>Amaranthus caudatus</i>	Amaranth	Choline, magnesium, PUFAs
<i>Psidium guajava</i>	Guava	Vitamin C
<i>Carica papaya</i>	Papaw	Vitamin C
<i>Chenopodium mexicanum</i>	Quelites	Magnesium, vitamin C
<i>Capsicum annuum</i>	Chili	Vitamin C
<i>Solanum lycopersicum</i>	Tomato	Lycopene, vitamin C
<i>Citrullus lanatus</i>	Watermelon	Lycopene
<i>Ictalurus punctatus</i>	Catfish	Vitamin D
<i>Thunnus albacares</i>	Tuna fish	Vitamin D
<i>Cucurbita pepo</i>	Squash seeds	PUFAs
<i>Cucurbita pepo</i>	Squash	Vitamins C and E
<i>Persea americana</i>	Avocado	MUFAs, vitamin E
<i>Lactobacillus spp</i>	Tejuino ¹ , Pulque ² , Tepache ³	Probiotics

Adapted from Ledesma-Solano *et al*^[126]. MUFAs: Monounsaturated fatty acids; PUFAs: Polyunsaturated fatty acids. ¹Tejuino, fermented maize beverage; ²Pulque, fermented agave plant beverage; ³Tepache, fermented fruit beverage, commonly pineapple.

is also true that globally, many populations are losing their food culture^[123].

On the other hand, commercial diets and international guidelines are intended for the general population; however, by means of nutritional genomics, the trend for the prevention and treatment of obesity-related liver diseases may now consider the individual's genetic make-up and environmental context. To date, individual genotyping is not feasible in all regions of the globe, so a personalized diet is an idea that still seems distant from application^[124]. However, based on what has been discussed in this paper, a country with knowledge of its genetic and food history and of the distribution of the selected nutrient-interacting genes related to ancestral diets, has the advantage of being able to appropriately adjust nutritional recommendations by regions^[125]. Thus, an alternative approach could be first to focus on a "region-tailored diet" as at present no effective pharmacological therapy exists for obesity/NASH^[99].

In particular, Mexico has been the origin of many endemic and domesticated plants and animals ever since pre-Hispanic times. A regionalized diet is feasible if it is based on local fresh produce, such as seasonal fruits and vegetables, grains and oilseeds that contain low-calorie nutrients and many functional ingredients as depicted in Table 5. In resemblance to the ancestral diet, regional diets could be rich in vitamins, minerals, and folates that are known today to avoid steatosis. The consumption of a high-starch diet rich in complex

carbohydrates instead of simple sugars is compensated for by a high number copy of the *AMY1* gene. The *ABCA1*, *CD36*, and *Apo E* genes speak of a diet low in animal fats, yet adequate in vegetal oils, and avoiding milk and dairy products may be essential. In general, these polymorphisms have a higher prevalence among the native populations; nonetheless, the mestizo population still shares much of its ancestral Amerindian component, indicating a traditional staple diet is still better for healthier nutrition.

Another benefit of the Mexican staple diet is the well-known combination of maize and nixtamalized (alkaline-treated) maize-derived products with beans. These foods not only provide essential amino acids, calcium and niacin^[45,126] but also act as natural prebiotics and add extra resistant starch required for a healthy gut metabolism. Moreover, these nutrients were supplemented with the consumption of indigenous fermented-beverages such as *tepache*, *pulque* and *tejuino* that provide complementary probiotics (Table 5). Other ingredients that are not considered in modern-day diets are the medicinal/ culinary plants that were often added to the food as species or herbs that are known to be beneficial.

CONCLUSION

Due to the high prevalence of obesity in Mexico and abroad, it appears feasible that any attempt to provide an intervention strategy should be based on the most frequent genetic polymorphisms and food culture of each population. This approach could provide a fitter gene-nutrient interaction that justifies the adoption of a regionalized diet, which is not only socially accepted by the general public, but is also energy-balanced, natural, and nutritious. In the age of globalization, it would only be fair to take advantage of the many Mesoamerican "gifts" that have been given to the world, such as maize, beans, tomatoes, squash, potatoes, vanilla, cocoa, and chili, instead of promoting an apparently well-balanced diet with industrial processed ingredients. Therefore, to combat obesity and its unhealthy consequences, it is crucial to continue analyzing the genetic signature "written" on the human genome. This action may be worth replicating in other populations around the world to achieve sustainable and healthier lifestyles according to the genetic background and food culture of each society.

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Management of hepatocellular carcinoma with portal vein thrombosis

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requires an understanding of multiple therapeutic options. PVT is present in 10%-40% of HCC at the time of diagnosis, and is an adverse prognostic factor. Management options are limited, as transplantation is generally contraindicated, and surgical resection is only rarely performed in select centers. Systemic medical therapy with sorafenib has been shown to modestly prolong survival. Transarterial chemoembolization has been performed in select cases but has shown a high incidence of complications. Emerging data on treatment of PVT with Y-90 radioembolization suggest that this modality is well-tolerated and associated with favorable overall survival. Current society guidelines do not yet specifically recommend radioembolization for patients with PVT, but this may change with the development of newer staging systems and treatment algorithms. In this comprehensive literature review, we present current and available management options with the relative advantages, disadvantages and contraindications of these treatment options with summarized data on overall survival.

Key words: Hepatocellular carcinoma; Portal vein thrombosis; Yttrium 90; Selective internal radiation therapy; Management

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Core tip: Management for hepatocellular carcinoma (HCC) with portal vein thrombosis (PVT) is more challenging and limited than for HCC without PVT. Currently, liver transplantation is generally contraindicated and surgical resection with curative intent is controversial. Systemic chemotherapy with sorafenib has been shown to modestly prolong survival. Transarterial chemoembolization has traditionally been considered to be contraindicated due to its high embolic effect causing hepatic necrosis and worsening liver dysfunction. External radiation therapy is limited by the sensitivity of the liver to radiation toxicity. In this review, these

Abstract

Management of hepatocellular carcinoma (HCC) with portal vein thrombosis (PVT) is complex and

treatment options are comprehensively presented, along with a relatively new modality in the treatment of HCC, selective internal radiation therapy with yttrium-90.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, the sixth most common cancer overall, and the third most common cause of cancer-related death worldwide^[1]. It is responsible for over 700000 deaths annually^[2,3]. In Western countries, the incidence of HCC is expected to increase in the coming years because of an aging cohort of patients infected with hepatitis C several decades ago and the rising epidemic of nonalcoholic fatty liver disease^[4-7].

Portal vein thrombosis (PVT) is a common complication of HCC, which is associated with a poor prognosis. Approximately 10%-40% patients with HCC have PVT at the time of diagnosis^[8-10], and approximately 35%-44% will be found to have PVT at the time of death or liver transplant^[11]. Patients with PVT are more likely to have metastatic disease at diagnosis, have fewer therapeutic options, and have shortened overall survival compared to patients without PVT. In patients with PVT treated with supportive care, studies have reported overall survival ranging from two to four months, compared to 10-24 mo in HCC patients without PVT^[9,10,12]. Thrombus involving the main portal vein is a worse prognostic factor than thrombus involving a branch portal vein^[13].

Management options for HCC with PVT are more limited than for HCC without PVT. Liver transplantation is generally contraindicated in these patients, and surgical resection with curative intent is controversial and not performed in most centers. Percutaneous ablation, another potentially curative therapy for small tumors, is less effective and potentially unsafe for tumors with PVT due to their proximity to the hepatic vascular structures. Transarterial chemoembolization (TACE) has traditionally been considered to be contraindicated in cases of PVT due to its high embolic effect and the potential for inducing hepatic necrosis and worsening liver dysfunction. External radiation therapy is limited by the sensitivity of the liver to radiation toxicity and the poor hepatic reserve of most HCC patients. These treatment options are reviewed below, along with a relatively new modality in the treatment of HCC, selective internal radiation therapy with yttrium-90, which is finding application in the treatment of HCC with PVT (Table 1).

SURGICAL MANAGEMENT

For eligible patients, liver transplantation remains the definitive curative treatment for cirrhosis as well as for hepatocellular carcinoma. However, due to high rates of tumor recurrence after transplantation in cases of HCC with PVT, transplantation is generally regarded as contraindicated in these patients^[14-16]. Surgical resection is often technically infeasible in patients with PVT, and is associated with poorer outcomes. In a series of 406 patients who underwent partial hepatectomy for HCC with PVT, the one- and three-year overall survival were 34% and 13%, respectively, and the corresponding disease-free survival rates were 13% and 5%^[17]. Another large series of 438 PVT patients who underwent resection for PVT found main portal vein tumor thrombus to be a significant risk factor for recurrence at 1 year, compared to branch portal vein (79% vs 45%)^[18]. Overall survival in this series was 18.8 mo with branch portal involvement and 10.1 mo with main portal involvement. Smaller series have reported overall survivals of 9 to 15 mo in selected patients, mostly with good underlying liver function, many of whom received additional treatments^[19-22]. These series generally report operative mortality rates of 0%-6%.

The most common staging system for HCC employed in American and European centers, the Barcelona Clinic Liver Cancer (BCLC) system, recommends against surgical resection in cases of PVT^[23]. Surgical resection for HCC with PVT is more frequently employed across Asia^[24], where hepatitis B is more common as a predisposing risk factor and patients tend to have better underlying liver function. Some centers have reported survival outcomes for patients with various degrees of portal vein invasion ranging from 9 to 33 mo^[13]. Outcomes of surgical resection for tumors involving the main portal vein remain relatively poor in these series, with reported median survival of nine to ten months, and 3-year survival rates of zero to six percent.

SYSTEMIC THERAPY

Sorafenib is an oral multikinase inhibitor that targets tumor cell proliferation and angiogenesis. It was the first systemic agent shown to improve overall survival in patients with unresectable HCC, including those with PVT, and it is currently the only therapy specifically recommended for HCC with PVT in American Association for the Study of Liver Disease (AASLD) and European Association for Study of the Liver (EASL) guidelines^[25,26]. The Sorafenib HCC Assessment Randomized Protocol (SHARP) trial^[27] compared sorafenib to placebo in patients with good baseline liver function (mostly Child-Pugh A) with advanced, unresectable HCC. Median survival in the treatment group was 10.7 mo compared to 7.9 mo in the

Table 1 Up-to-date summary of management options for hepatocellular carcinoma with portal vein thrombosis

	Survival data (mo)					Adverse effects	Key references	Additional comments
	Overall survival	Main PVTT	Branch PVTT	CP-A	CP-B			
Supportive care	2-4						Schoniger <i>et al</i> ^[12] , Minagawa <i>et al</i> ^[9] , Llovet <i>et al</i> ^[10]	
Surgical resection	9-33	9-10				0%-6% operative mortality	Lau <i>et al</i> ^[13] , Shi <i>et al</i> ^[17] , Chen <i>et al</i> ^[18] , Lin <i>et al</i> ^[21]	Employed in select centers
Sorafenib	6-8			8.1		skin reaction, diarrhea, fatigue	Llovet <i>et al</i> ^[27] , Cheng <i>et al</i> ^[29]	Recommended by AASLD and EASL guidelines; Dose reduction in 25%, interruption in 44% Investigational
XRT	9.6					radiation induced liver disease	Toya <i>et al</i> ^[53]	
TACE	7-10	5.3	10.2	7.4	2.8	liver failure, postembolization syndrome	Pinter <i>et al</i> ^[40] , Chung <i>et al</i> ^[41] , Luo <i>et al</i> ^[43] , Xue <i>et al</i> ^[48]	Lowest risk with nonocclusive thrombus, cavernous transformation, superselective TACE
Y-90 SIRT	5-17	9	17	10.4	5.6	fatigue, hyperbilirubinemia, GI ulceration	Salem <i>et al</i> ^[70] , Hilgard <i>et al</i> ^[69] , Sangro <i>et al</i> ^[71]	Currently, PVT is one of the indications for Y90

control group. In a subgroup analysis^[28], patients with macroscopic vascular invasion, presumably largely consisting of PVT, had an overall survival of 8.1 mo in the sorafenib group, compared to 4.9 in the control group. The respective times to progression were 4.1 and 2.7 mo. Both of these differences were significant. The Sorafenib Asia-Pacific Trial, the other landmark trial of oral sorafenib for patients with advanced stage HCC, obtained largely concordant results^[29]. Sorafenib was found to prolong overall survival in all patients with unresectable HCC (6.5 mo vs 4.2 mo). In subgroup analyses^[30], sorafenib was found to have modestly prolonged survival in patients with macroscopic vascular invasion and/or extrahepatic spread of tumor (5.6 vs 4.1). Time to progression was likewise somewhat prolonged (2.7 mo vs 1.2 mo).

Subsequent studies have confirmed that sorafenib confers a relatively similar survival benefit to patients with PVT compared to those without, with a similar safety profile^[31]. The most frequent adverse reactions to sorafenib are hand-foot skin reaction, diarrhea, and fatigue, which necessitate dose reduction or discontinuation in a minority of patients.

Sorafenib is considered appropriate for patients with unresectable HCC whose liver disease remains well-compensated (Child-Pugh A). A portion of Child-Pugh B patients may benefit from sorafenib^[32], however Child-Pugh C patients are unlikely to benefit from sorafenib due to their limited life expectancy and inability to tolerate the medication^[33]. Treatment is generally continued until there is evidence of disease progression or death. Combination of sorafenib with locoregional therapies remains an area of active investigation. Besides sorafenib, multiple additional agents are under investigation, but so far none have demonstrated efficacy in phase III trials, either in the setting of progression on sorafenib or as primary

therapy^[34]. Although a select group of patients responds remarkably to sorafenib, even to the point of downstaging^[35,36], the majority of patients with PVT have relatively short overall survival expectancy despite treatment, which has inspired continued efforts at developing locoregional therapeutic options.

TRANSARTERIAL CHEMOEMBOLIZATION

TACE is a percutaneous technique for delivering chemotherapeutic agent (generally either cisplatin or doxorubicin) directly to a liver tumor *via* its arterial blood supply. The drug is suspended in iodized ethyl esters of poppyseed oil (lipiodol), or impregnated into drug-eluting beads, and is then delivered directly into the feeding tumoral artery. TACE takes advantage of the fact that HCC is preferentially fed by the hepatic arterial circulation, while the majority of blood flow to the normal liver comes from the portal vein, which allows relatively selective targeting of tumor and sparing of uninvolved liver. TACE has an established role as a locoregional therapy for inoperable tumors, which has been shown to prolong survival^[37-39], and as a means of maintaining local control of tumor while a patient awaits definitive surgical management, the so-called "bridge to transplant"^[26].

Historically, PVT has been considered a contraindication to TACE due to the risk of precipitating liver necrosis and worsened liver dysfunction, related to the embolic effect of TACE on an already compromised hepatic vascular supply. In more recent years, several groups have reported that subselective and superselective TACE can be performed safely in some patients with PVT, and is associated with improved overall survival^[40-47]. Overall survival among PVT patients treated with TACE in these studies ranged from 7.0 to 10.2 mo. In a large nonrandomized study, Luo

and colleagues prospectively treated 164 patients with PVT with either lipiodol TACE or conservative treatment^[43]. Twelve and 24 mo survival rates in the TACE group were significantly prolonged (30.9% and 9.2%, vs 3.8% and 0%), and the benefit was consistent across patients with segmental and main PVT. A 2013 meta-analysis examined eight controlled trials involving 1601 patients with PVT^[48]. TACE was favored over conservative treatment in all studies, and pooled analysis estimated TACE to have a significantly beneficial effect on 6 mo and 1 year mortality (HR = 0.41 and 0.44, respectively). In this analysis, TACE was favored for main as well as branch portal vein tumor thrombus, and in both Child-Pugh A and B cirrhotics, although there were fewer patients and more heterogeneity in these comparisons. A 2014 meta-analysis of 5 studies involving 600 patients likewise found TACE to be associated with improved 1-year survival compared with placebo in patients with PVT^[47].

Overall, TACE is now regarded as a viable therapeutic option for select patients with PVT, especially for those with nonocclusive thrombus or cavernous transformation of the portal vein, provided their underlying liver function is relatively preserved and their tumor burden is such that the procedure is technically achievable. However, reported overall survival of 7.4 to 10.2 mo is only marginally better than systemic sorafenib, and inferior to survival that has been reported with other modalities, in particular selective internal radiation therapy.

EXTERNAL RADIATION THERAPY

Use of external radiation therapy for liver lesions has traditionally been limited in patients with compromised underlying liver function. These patients are especially prone to develop radiation-induced liver disease, in the form of hepatic veno-occlusive disease^[49,50]. However, newer techniques, in the form of stereotactic body radiation therapy, allow high doses of radiation to be delivered very selectively, with relative sparing of uninvolved liver^[51,52].

There have been few studies specifically examining the effect of external radiation therapy in HCC with PVT. Toya and colleagues^[53] achieved a median survival of 9.6 mo in 34 HCC patients with PVT using conformal radiation therapy. Lee and colleagues treated 46 patients with PVT with conformal radiation therapy and reported complete or partial response in 33%^[54]. In this series, patients who initially responded to treatment showed a 1-year survival of 66.8%, compared to 27.4% among nonresponders. Other groups have reported overall survival of 10 mo or more in these patients when external radiation therapy is combined with other modalities^[55-57], and some studies have specifically combined radiation with sorafenib^[58,59] and TACE^[60-63]. A recent retrospective series of 97 patients compared radiotherapy to systemic

sorafenib in patients with PVT, and found that, after performing propensity score matching, radiotherapy was associated with longer overall survival^[64]. Use of external radiation therapy for HCC is not yet regarded as standard treatment, but remains an area of active investigation.

SELECTIVE INTERNAL RADIATION THERAPY

Selective internal radiation therapy (SIRT) or trans-arterial radioembolization with yttrium-90 is a relatively new therapeutic modality for HCC and other liver tumors, in which therapeutic doses of radiation are delivered to the tumor transarterially. There are two commercial products currently available, SIR Spheres, which are 20-60 μ m particles made of a biocompatible resin, and Theraspheres, which are 20-30 μ m glass particles. Both are considered permanent embolic agents, although due to their small size have much less embolic effect than a TACE procedure, with less effect on hepatic vascular dynamics^[65]. Indeed, continued blood flow to treated tissue is necessary and desirable for radiation to have its intended effect through the production of free radicals. Yttrium-90 is a pure beta-emitting isotope that decays to zirconium-90 with a half-life of 64.1 h. Ninety-four percent of the total radiation dose is delivered within 11 d of the procedure. The emitted radiation penetrates surrounding liver tissue to an average depth of 2.5 mm and a maximum depth of 11 mm, such that there is essentially no expected radiation exposure to non-treated individuals in contact with the patient, and post-procedure isolation precautions are not necessary. Radiation doses delivered to the tumor, however, can be very high due to preferential flow of embolic particles toward hypervascular tumor tissue, in a ratio of between 3:1 and 20:1 compared to unaffected liver^[66]. Particles preferentially accumulate in the periphery of tumor masses, where most viable tumor cells are located. On the basis of explant studies, it has been estimated that local radiation doses on a microscopic scale may vary from 100 Gy to more than 3000 Gy^[66]. The radiation dose may be delivered to the whole liver, to both lobes sequentially, to a single lobe, or to a segment.

SIRT has found application as a locoregional therapy for unresectable HCC that is not amenable to TACE because of diffuse or multifocal disease, or as an alternative to TACE^[13,67]. Although no randomized controlled trials have been performed directly comparing SIRT with TACE or other local therapies, numerous retrospective series have reported favorable outcomes and acceptable safety profiles in HCC patients^[68-71]. Subgroup analyses from the three largest series of HCC patients treated with SIRT, together totaling over 700 patients, 234 of whom had PVT, demonstrated remarkably similar overall survival

times ranging from 10.0 to 10.4 mo among all patients with PVT^[68-71]. The largest group of PVT patients, reported by Salem and colleagues, showed overall survival of 16.6 mo among Child-Pugh A cirrhotics with branch PVT, decreasing to 4.5 mo among Child-B cirrhotics with main PVT^[70]. This and other series have reported better overall survival in patients who demonstrate complete or partial response by WHO or EASL criteria following SIRT. Smaller series of patients with PVT treated with SIRT have demonstrated largely concordant results, with overall survival ranging from 7.2 to 13 mo^[72-75]. A recent prospective phase II trial including 35 patients with branch or main PVT treated with SIRT has reported an overall survival of 13 mo^[76]. In this study, Child-Pugh A patients showed an overall survival of 16 mo, compared to 6 mo for Child-Pugh B patients. A small nonrandomized study compared outcomes in 32 patients with unresectable HCC, one half of whom had major vascular invasion, following either TACE or SIRT^[77]. Among patients with major vascular invasion, the SIRT group showed an overall survival of 12.0 mo, compared to 8.0 mo in the TACE group.

Toxicity of SIRT is generally mild compared to TACE. A robust post-embolization syndrome with fever, abdominal pain and elevated liver enzymes, such as is common after TACE, is infrequently seen. The most common side effects are fatigue (occurring in approximately 40% of treated patients) and elevated bilirubin (in approximately 20%)^[78]. Most serious complications, including radiation pneumonitis, radiation cholecystitis, hepatic abscess, and radiation induced liver disease are reported in < 1% of patients. Gastrointestinal ulceration has been reported to occur in approximately 5% of patients^[78], but several recent large series have reported a 0% rate of GI ulceration^[70,76], and this complication may be largely avoidable with careful pre-procedure preparation and appropriate quantitative radiation dosing^[79]. SIRT is commonly performed as an outpatient procedure, unlike TACE which usually requires at least an overnight hospital admission. However, SIRT does require a separate prior mapping procedure, consisting of mesenteric angiography to ensure that there are no branching vessels near the intended catheter position, such as the gastroduodenal artery or left gastric artery, which could result in off-target embolization to bowel. If these vessels are identified they may be preemptively coil-embolized. Generally as part of the same pre-SIRT mapping procedure, technetium-labelled macroaggregated albumin is injected from the intended catheter position, and subsequent scintigraphic or SPECT imaging is performed to quantify the fraction of embolic particles that are shunted to the lungs. The accepted safe radiation dose to the lungs is < 30 Gy in a single procedure, and < 50 Gy total over multiple procedures. Inability to prevent excessive lung dose or off-target embolization are contra-indications to SIRT. Additionally, ideal

candidates for the procedure will have good ECOG performance status (≤ 2), relatively preserved liver function (bilirubin < 2, albumin > 3, platelets > 50), and adequate renal function (creatinine < 2)^[78,80].

GUIDELINES FOR MANAGEMENT OF PORTAL VEIN TUMOR THROMBUS

The BCLC staging system regards portal vein invasion as advanced (stage C) disease, for which systemic therapy in the form of sorafenib is the recommended treatment^[23]. Current guidelines from the AASLD^[26] and the EASL^[81] largely embrace BCLC staging and treatment recommendations. AASLD guidelines recognize radioembolization as an effective treatment, but stop short of recommending it for any specific HCC-related indication due to lack of data directly comparing it to alternatives such as TACE or sorafenib. Current EASL guidelines discourage TACE for patients with macroscopic vascular invasion, and state that radioembolization can be safely performed on patients with PVT with promising results, but more study is needed before it can be recommended as standard therapy. 2015 guidelines from the National Comprehensive Cancer Network state that sorafenib and locoregional therapy are both options for patients with unresectable disease who are not transplant candidates, but that arterially directed therapies are relatively contraindicated in patients who have main portal vein thrombosis^[82]. Resection for patients with major vascular invasion is described as controversial, but may be considered.

FUTURE DIRECTIONS

There are a number of staging systems to characterize HCC^[25,83-87]. The BCLC system has been widely adopted due to its robust prognostic and therapeutic validation. However, as therapeutic options for HCC, particularly for those patients with PVT, continue to evolve, limitations of the BCLC system have become evident. All patients with macroscopic vascular invasion are considered to have advanced, stage C disease, and are recommended for systemic treatment. Given the data on other surgical and locoregional treatments reviewed above, it is likely that this recommendation will come to be regarded as too limiting. The recently published Hong Kong Liver Cancer (HKLC) staging system^[88] is based on a cohort of 3856 patients, and was developed using rigorous statistical modeling. This system separates extrahepatic from intrahepatic vascular invasion, and generally recommends more aggressive management of early and intermediate disease, which is likely more in line with current and evolving practice in specialized centers. The HKLC staging system may represent an important step in classifying HCC and guiding treatment, but requires

further validation, including in Western cohorts, before it is likely to be adopted in major guidelines.

An active area of investigation concerns the combination of sorafenib with locoregional therapies such as TACE and SIRT^[89]. This combination may maximize tumor cell killing by preventing compensatory revascularization in response to proangiogenic factors elaborated by ischemic tumor cells. Subgroup analyses of the SHARP and Asia-Pacific trials both found sorafenib to be beneficial in patients who had received prior TACE^[28,30], however these patients received sorafenib long after their TACE procedure. The two largest randomized controlled trials to combine TACE and sorafenib, involving 458 and 307 patients with unresectable HCC randomized to receive sorafenib or placebo following TACE, reported only modest benefits associated with the addition of sorafenib^[90,91]. However, a smaller randomized controlled trial has shown a significant survival benefit^[92], and nonrandomized series have likewise shown promising results^[93-98]. These studies used varying protocols for combining TACE and sorafenib. Some, including the two largest, excluded patients with PVT. The ongoing START trial is a phase II prospective study of the effect of combined TACE and sorafenib in patients with good performance status and mostly BCLC B tumors, although second order branch portal vein involvement was allowed. In an interim analysis of 147 patients^[99], adverse events appeared similar to those associated with the treatments independently, and early outcomes data appeared encouraging. Overall, the safety and efficacy of combined TACE and sorafenib in the population of patients with PVT remains to be determined.

Fewer studies have focused on the combination of SIRT with sorafenib. A recently published phase II trial of 29 patients with BCLC stage B or C disease treated with yttrium-90 SIRT followed by sorafenib initiated 14 days post procedure, reported similar rates and severity of treatment-related adverse events as would be expected with the treatments separately^[100]. Importantly, eligibility for treatment with sorafenib, whether in the context of a trial or in routine clinical use, requires that the patient's liver function be maintained, ideally at the Child-Pugh A level. A recent series of 63 patients with PVT and Child-Pugh score ≤ 7 treated with yttrium-90 SIRT found that progression of Child-Pugh A to Child-Pugh B disease at the time of tumor progression following SIRT occurred in 55% of patients^[101]. It may therefore be prudent to initiate therapy with sorafenib relatively soon after the procedure, rather than waiting until the time of tumor progression, to derive the maximum survival benefit before the patient's underlying liver function deteriorates to the point where sorafenib is contraindicated. The safest and most effective combination of TACE, SIRT and sorafenib in PVT and in HCC generally remains an area of active investigation, with several ongoing clinical trials^[89]. Additionally, data from the ongoing GIDEON study, a global observational

database of HCC patients treated with sorafenib, may likewise yield insights into the safety and efficacy of various combinations of therapies in the real-world clinical setting^[102,103].

CONCLUSION

HCC is a significant source of worldwide morbidity and mortality, and one that is likely to increase in prevalence in Western countries in the coming years. Despite the emergence of numerous effective, life-prolonging treatments for HCC, patients with PVT remain especially challenging to treat and continue to experience shortened survival. Orthotopic liver transplantation is generally contraindicated in these patients due to high rates of recurrence. Hepatic resection with curative intent is controversial and infrequently employed in American and European centers, but may offer favorable overall survival in selected patients, especially those with branch portal vein involvement and good liver function. In patients who are not surgical candidates, various therapies including systemic sorafenib, TACE, and yttrium-90 SIRT may be management options. Of these, SIRT has demonstrated excellent safety and tolerability, and a growing body of data supports its use in patients with PVT.

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Lessons from rare tumors: Hepatic lymphoepithelioma-like carcinomas

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Abstract

In this review we focus on lymphoepithelioma-like hepatocellular carcinomas (LEL-HCC) and lymphoepithelioma-like cholangiocarcinomas (LEL-ICC). Despite their rarity, these tumors are of general interest because of their epidemiological and clinical features, and because they represent a distinct model of interaction between the immune system

and neoplastic cells. Approximately half of LEL-HCC arise in the context of chronic hepatitis C virus (HCV) infection and have been described both in Eastern and Western patients. By contrast, LEL-ICC is associated in almost all cases with Epstein-Barr virus (EBV) infection and exhibits the same epidemiological features of EBV related malignancies. Compared with classical hepatocellular carcinoma and intrahepatic cholangiocarcinoma of corresponding stage, both LEL-HCC and LEL-ICC are characterized by lower rates of recurrence after surgery and better overall survival. How this behavior is related to distinct genetic alterations and tumor microenvironment is unclear. The pathophysiological mechanisms of lymphoid infiltrations seem to be different among the two groups of tumors. In fact, LEL-HCC frequently arises in the context of inflammatory changes driven by HCV infection, and has been recognized as a variant of classical hepatocellular carcinoma. At variance, lymphocyte recruitment of LEL-ICC is similar to that described in nasopharyngeal carcinoma and gastric LEL, and possibly depends on the expression pattern of latent EBV infection.

Key words: Lymphoepithelioma; Lymphoepithelioma-like carcinoma; Nasopharyngeal carcinoma; Lymphoepithelioma-like hepatocellular carcinoma; Lymphoepithelioma-like intrahepatic cholangiocarcinoma; Epstein-Barr virus infection

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Core tip: Despite their rarity, lymphoepithelioma-like hepatic carcinomas are of general interest because of their peculiar epidemiological and clinical features, and because they represent a distinct model of interaction between the immune system and neoplastic cells. Compared with classical hepatocellular carcinoma and intrahepatic cholangiocarcinoma of corresponding stage, lymphoepithelioma-like hepatic carcinomas are characterized by lower rates of recurrence after surgery and better overall survival. Whether these differences

are related to distinct genetic alterations or to the tumor microenvironment is unclear. Here we review the features of these tumors and the mechanisms of lymphoid infiltration.

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INTRODUCTION

The term lymphoepithelioma denotes a subgroup of nasopharyngeal carcinomas. These tumors are mostly related to Epstein-Barr virus (EBV) infection, and are characterized by the concomitance of undifferentiated carcinoma cells and polyclonal lymphocyte infiltration^[1-4]. Tumors with similar morphological features, lymphoepithelioma-like (LEL) carcinomas, have been described in salivary glands^[5], lungs^[6], thymus^[7], stomach^[8], colon^[9], uterus^[10], ovaries^[11], bladder and urinary tract^[12], breast^[13], and skin^[14]. Although the association with EBV infection was confirmed in LEL carcinomas of the digestive tract^[15], lung^[16,17], and thymus^[18], it was not found in LEL carcinomas of the breast^[19] and uterus^[20]. In the liver, this type of tumor is extremely rare. Thus far, 29 cases of LEL hepatocellular carcinoma (HCC) and 24 cases of LEL intrahepatic cholangiocarcinoma (ICC) have been described.

LEL-HCC

In 24 of 29 cases^[21-29], the diagnosis of LEL-HCC was based on the retrospective analysis of three different series: one from Japan^[21], one from France^[22], and one from the United States^[23]. These studies were conducted in different periods and differ substantially in design and patient clinicopathological features. Considering these caveats, all three of the studies suggest that patients with LEL-HCC have a better outcome compared with patients with classical hepatocellular carcinoma. The study from Japan dates back to 1994 and was aimed at evaluating the features and the long-term outcome of 11 hepatocellular carcinomas heavily infiltrated by inflammatory cells. The investigation was limited to tumors of less than 3 cm in diameter. The rate of recurrence at 5 years after surgery was 9.1 percent in these patients and 47.5 percent in 116 controls matched for the etiology of the associated liver disease and tumor size. The study from France evaluated the features and the disease course after liver transplantation in 5 patients with hepatocellular carcinoma with lymphoid infiltration. The 5-year survival was significantly better when compared with 163 patients transplanted for

hepatocellular carcinoma. Finally, the study from the United States compared 8 cases of resected tumors classified as inflammatory hepatocellular carcinoma with 18 undifferentiated hepatocellular carcinomas. The rates of local recurrence and distant metastases were 25% and 33.3% respectively, for those patients with inflammatory hepatocellular carcinoma, and 12.5% and 22.5%, respectively for those patients with undifferentiated hepatocellular carcinoma. When the survival data from all 29 patients with LEL-HCC were pooled together, it was evident that 19 patients were alive and free of disease 15 mo to 10 years after surgery (median: 43 mo). Six patients were alive with disease recurrence, and 4 patients died of recurrent disease.

The retrospective design of the above studies implicates that the pre-operative features of these patients are largely unknown. In particular, descriptions of the radiological findings are available in only two cases. In one patient, both computed tomography (CT) and magnetic resonance imaging (MRI) scans showed the features of classical hepatocellular carcinoma, as enhancement in the arterial phase and wash-out in the portal phase were observed^[24]. In the second patient, the CT scan did not show arterial enhancement, whereas the contrast phase of MRI showed peripheral rim enhancement and hyper-intensity of the nodule in T2 sequences^[25]. LEL-HCC was associated with liver cirrhosis in 13 cases, with hepatitis C virus (HCV) infection in 16 cases, hepatitis B virus (HBV) in 3 cases, and EBV in 1 case. Histopathological analysis of LEL-HCC showed that in 16 cases the tumors were poorly differentiated (Figure 1). Well-differentiated or moderately-differentiated tumors were observed in the remaining cases. Interestingly, in 3 cases the same tumor exhibited different grades of differentiation. Positivity for HepPar 1 was described in 3 of 3 tested cases. Cytokeratin 7 and 19 were positive in 4 cases. The overexpression of p16 protein was found in 2 cases. Nests of epithelial cells were surrounded by polyclonal lymphoid cells. Lymphocyte subset analysis showed that CD3-positive cells were 10 times more frequent than B cells. The majority of T cells were CD8-positive in 13 cases, whereas FoxP3 positive cells represented a minority in the single tested case. Remarkably, and similar to the metastases of undifferentiated NPC, the nodal metastatic lesions from LEL-HCC did not show lymphocytic infiltration. The molecular changes associated with LEL-HCC are unknown.

LEL-ICC

Twenty-two of the 24 patients with LEL-ICC were from South-East Asia. In seven of them, the diagnosis was made in surgically resected patients in a single center in Hong-Kong, between 1999 and 2008, and accounted for 5% of all ICC in that period^[30]. The 5-year survival rate of these patients was 100 per cent

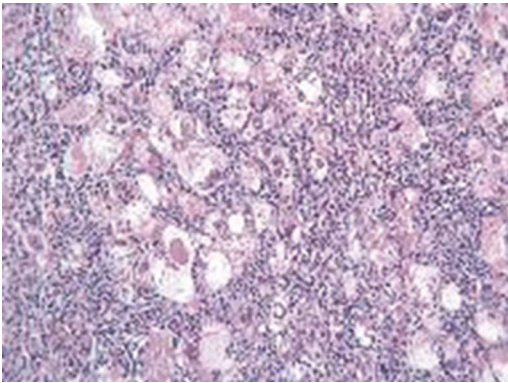


Figure 1 Histological picture of lymphoepithelioma-like hepatocellular carcinoma. Undifferentiated neoplastic cells are surrounded by lymphoid stroma (magnification $\times 250$) (Obtained from Nemolato *et al.*^[29], reprinted with permission).

and was significantly better compared with the 13.2 percent observed in 11 matched controls (classical cholangiocarcinomas). Five cases were from a single center in Taiwan^[31]. The remaining patients were from a single case report^[32-42]. At the time of writing, fifteen patients were alive without disease 2 to 165 mo after surgery (median recurrence free survival: 39 mo), and three patients were alive with recurrent disease (median survival: 56 mo). In addition, four patients died of recurrent disease (median survival: 48 mo), one patient died of post-operative complications and, in another case, no information on survival is available. Descriptions of the CT scans were available only in five cases and were limited to the unenhanced phase^[30,32,35,42]. In these cases, hypodense lesions ranging from 3 to 10 cm in diameter were described. Dynamically enhanced MR T1 imaging was available only in one case, and (similar to ICC) showed centrifugal enhancement of the neoplastic lesion^[30]. LEL-ICC was associated with cirrhosis in 6 cases. HBV infection was documented in 6 cases and HCV infection in two cases. EBV infection was found in 17 cases. Histopathology showed the features of adenocarcinoma with different grades of glandular differentiation and polyclonal lymphocytic infiltration. Interestingly, in at least seven cases, two different components were recognized within the tumor^[31,32,34,37,38,40]. One component consisted of undifferentiated lymphoepithelioma-like cholangiocarcinoma, whereas the other consisted of an adenocarcinoma without lymphocytic infiltration. CK AE1/A3, CK7, and CK19 immunoreactivity was detected in all of the cases but one. The latter tumor was in fact HepPar1 positive, raising the possibility of a misdiagnosed HCC, or a combined hepatocellular and cholangiocarcinoma^[38]. Immunolabeling for markers of stemness, such as CD133 and EpCam, was positive in 5 of 5 evaluated cases^[30]. In situ hybridization for detection of EBV non coding RNA, using antisense oligoprobes, was positive within the epithelial cells in 17 cases. Lymphocytic infiltration consisted of CD3 and CD20 positive cells,

Table 1 Characteristics of the patients with lymphoepithelioma-like hepatic carcinomas *n* (%)

	LEL-HCC (<i>n</i> = 29)	LEL-ICC (<i>n</i> = 24)
Age at diagnosis, median (range)	61 (39-79)	53 (19-71)
HBV infection (positive)	3	7
HCV infection (positive)	16	2
EBV infection (positive)	3	17
Cirrhosis	13	6
Maximum diameter of the tumor (mm)	25 (13-130)	45 (16-160)
Surgical treatment (OLT/resection)	6/23	0/24
Rate of recurrence	6 (20.6)	8 (36.3)

LEL-HCC: Lymphoepithelioma-like hepatocellular carcinomas; LEL-ICC: Lymphoepithelioma-like cholangiocarcinomas; EBV: Epstein-Barr virus; OLT: Orthotopic liver transplantation; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

and was consistently negative for markers of active or latent EBV infection. One single study^[30] addressed the gene methylation status and the mutations of KRAS and EGFR genes in LEL-ICC. Loci coding for CRBP I (cellular retinol binding protein I) and CRBP IV (cellular retinol binding protein IV) showed significantly higher methylation status than in ICC. This finding is in agreement with the epigenetic changes occurring in EBV-related nasopharyngeal carcinoma. Wild-type KRAS and EGFR genes were detected in all of the cases.

INTRAHEPATIC LYMPHOEPITHELIOMA-LIKE CARCINOMA: WORKING HYPOTHESES

Hepatic LEL carcinomas, similar to gastric and lung LEL carcinomas, are characterized by a significantly better survival than classical HCC and ICC counterparts of corresponding stage. Whether the more favorable outcome of hepatic LEL carcinomas depends on distinct genetic and epigenetic changes, or whether the tumor infiltrating lymphocytes play a prominent role in improving the outcome of these patients remains unclear. In turn, the baseline features and the outcome of hepatic LEL carcinomas are also related to the clinical setting in which the tumor arises. Notably, the patients with LEL-HCC and LEL-ICC (Table 1) differ with regard to age, tumor size, presence of cirrhosis, and rate of HCV and EBV infections. The World Health Organization recently recognized LEL-HCC as a variant of HCC^[43]. Indeed, HCC is characterized by different grades of lymphoid infiltration^[44,45] and LEL-HCC represents an end of this spectrum. The molecular mechanisms linking inflammation and cancer depend on the complex network of chemokine and cognate receptor axes^[46-53]. In the setting of HCC, it has been shown that the CXCL12^[54,55] CXCL8^[56], CCL3^[57] CCL20^[58] and CCL22^[59] ligands, and the aberrant expression of their receptors affect tumor development and progression, angiogenesis and metastasis. Several

Table 2 Pathological findings in patients with lymphoepithelioma-like-hepatic carcinomas

	LEL-HCC (n = 29)	LEL-ICC (n = 24)
Histological features		
Poorly differentiated	16/29	10/24
Moderately differentiated	13/29	3/24
Combined LEL-HCC and HCC	None	
Combined LEL-ICC and ICC		9/24
Immunohistochemistry		
HepPAR1	3/3	1/2
CK7 and CK19	4/11	24/24
EpCAM	ND	7/7
CD133	ND	7/7
EBV status		
EBERs	3/18	17/24
LMP1/2 antigens	ND	0/8
LMP1 gene	ND	2/2
EBNA2	ND	1/1
Lymphoid infiltration		
CD3/CD20 ratio > 1	29/29	4/4
CD4/CD8 ratio < 1	11/29	1/4

LEL-HCC: Lymphoepithelioma-like hepatocellular carcinomas; LEL-ICC: Lymphoepithelioma-like cholangiocarcinomas; EBV: Epstein-Barr virus; EBERs: EBV-encoded RNAs; LMP: Latent membrane protein; EBNA2: Epstein Barr nuclear antigen 2; ND: Not done.

lines of evidence, however, note that the chemokine system also has a role in tumor control. Myeloid cell infiltration is associated with a poor prognosis, whereas T helper 1 infiltration directly correlates with a reduced risk of tumor recurrence^[60]. In addition, a pro-inflammatory microenvironment characterized by high expression of the innate immune genes TNF, IL6, and CCL2 is a predictor of survival^[61]. A validated model of a 14 immune gene signature, in patients resected for early HCC, is also associated with a better prognosis^[62]. Among the genes of this signature with increased expression of particular interest include CXCL10, CCL5, and CCL2. These chemokines are related with Th1 and NK cell recruitment. Potentially tumors with the features of LEL-HCC are characterized by a similar chemokine profile.

LEL-HCC arise in nearly half of the cases in the context of HCV-related cirrhosis. In these patients, chemokine-driven inflammatory changes related to HCV chronic hepatitis may play a role in LEL-HCC development and in lymphocyte recruitment^[63-66]; however, LEL-HCC also arises in apparently normal livers. Whether lymphocyte recruitment is driven by the same genetic alterations in both groups of LEL-HCC remains unknown.

In comparison with LEL-HCC, LEL-ICC contains a higher proportion of tumorous cells with features of stemness. Indeed, 7 of 7 samples were positive for EpCam and CD133 (Table 2). In addition, in at least 2 cases the tumor exhibited the features of hepatocellular and cholangiocarcinoma. In contrast to LEL-HCC, LEL-ICC is associated in almost all cases with EBV infection and exhibits the same epidemiological features of EBV related

malignancies^[67]. Consequently, the mechanistic of LEL-ICC should be analyzed considering this background. EBV infection is detected in nearly all patients with endemic Burkitt lymphoma^[68]. By contrast, the association of EBV infection with other malignancies such as Hodgkin and non-Hodgkin lymphomas^[69], post-transplant lymphoproliferative disorders^[70], gastric adenocarcinomas^[71], leiomyosarcomas^[72], and NPC^[73] is variable and is influenced by several factors, including age of infection, ethnicity, genetic susceptibility, socio-economic status, and immune function^[74,75]. Intriguingly, EBV-related NPC has a geographical and racial distribution similar to that of intra-hepatic LEL-ICC. In contrast to LEL-ICC, the genetic alterations occurring in NPC have been extensively studied^[49-53,76-80]. Several chromosomal abnormalities, including copy number changes on chromosomes 3p, 9p, 11q, 12p and 14q, and gene alterations, such as CDKN2A deletion and LTBR (lymphotoxin beta receptor) amplification, together with epigenetic changes, such as RASSF1A and TSLC promoter hypermethylation, have been described. According to the Catalogue of Somatic Mutations in Cancer (COSMIC), the most frequent mutations in NPC affect the CDKN2A gene (11% of tested samples), which encodes for the p16INK4A protein, and PIK3CA^[81]. The prominent lymphocyte infiltration of NPC is a model of the complex interactions between tumor and immune system^[82-86]. Most of the infiltrating lymphocytes consist of CD3 positive T cells; among them, the majority show the morphology of small resting lymphocytes. The ratio of CD4 to CD8 ranges from 0.4 to 2.2. Regulatory CD4 and CD25^{high} and FoxP3 positive cells represent 12% of all T cells. Two distinct subpopulations of CD8 positive lymphocytes were described: pro-inflammatory interleukin (IL)-17 secreting cells, and regulatory CD8 cells, whereas NK and B lymphocytes represent a minority of the tumor infiltrating lymphocytes. In addition, it has been shown that malignant NPC cells constitutively produce several cytokines, including IL-1 α , IL-1 β , and IL-18^[87,88], and chemokines including CCL20 and CXCL10^[89,90]. Infiltrating leucocytes in turn further amplify the inflammatory process by positive regulatory loops. Clinical and experimental findings indicate that NPC associated lymphocytes do not control tumor growth. Conversely, inflammation promotes tumor progression, as cytokines behave as tumoral growth factors. Immune escape is related to CCL20 dependent Treg expansion^[89] and resistance to interferon γ as a result of small EBV-encoded RNAs (EBERs) and latent membrane protein (LMP) 2A and B secretion^[91]. The complex interactions described in NPC possibly differ in other types of LEL carcinomas, depending on the burden of EBV intermediates and the latent gene expression. Three forms of EBV latency have been recognized^[92,93]. Latency I is characterized by the expression of Epstein Barr nuclear antigen (EBNA) 1, EBERs, and *BamHI* A rightward transcripts (BARTs). This expression pattern is found in Burkitt

lymphoma^[94]. By contrast, latency II is characterized by the variable expression of LMP1, LMP-2A and LMP-2B, BARTs, and EBERs. This pattern is typical of NPC^[95,96]. Latency III is characterized by the expression of EBNA 1, EBNA2, EBNA3, EBNA3B, EBNA3C, EBNA LP, LMP-1/2, BARTs, and EBERs. This expression pattern is found in isolated cell lines, and in the elderly with lymphomas^[97,98]. Finally, viral reactivation from latency expresses the *Bam*HI Z leftward open reading frame^[99]. In the setting of LEL carcinomas, the evidence that the lymphoid stroma is related to the pattern of EBV latency is provided by the finding that gastric LEL carcinoma, expressing EBNA1 mRNA, BARTs and LPM2, was associated with oligoclonal CD8 positive EBV specific lymphocytes^[100]. The evaluation of the EBV pattern of latency in LEL-ICC has been incompletely elucidated. EBERs were positive in 18 of 18 samples, LMP was negative in 8 of the samples by immune-histology^[30,40], and the LMP-related gene showed a 30 bp deletion in 2 of 2 cases^[35,36]. The *EBNA2* gene was found in one of one tested case. Progression of LEL-ICC is possibly associated with further molecular changes. In this respect, it is of interest that in more than one third of LEL-ICC cases, the tumor consisted of LEL-ICC and ICC with different grades of differentiation, and metastases of LEL-ICC usually lost the capacity to recruit inflammatory cells. These histological features implicate the concomitance of two different neoplastic clones. In contrast to HCC, little is known regarding the chemokines associated with ICC. Thus far, the single relevant finding in this regard is the overexpression of CXCL5^[101]. In ICC this chemokine has been associated with tumor progression and metastasis. The chemokines involved in HCC- LEL and in ICC-LEL are unknown.

The features of intrahepatic LEL carcinomas and their molecular differences compared with classical HCC and ICC warrant further study. Analysis of archival samples and prospective whole genome analysis of new cases would provide new insights into this issue. Given their rarity, addressing the above issues would be feasible only through a close cooperation between all the centers following these patients *via* the generation of a dedicated registry and the commitment of high profile basic researchers.

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Hepatitis C virus-specific cytotoxic T cell response restoration after treatment-induced hepatitis C virus control

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Abstract

Hepatitis C virus (HCV)-specific cytotoxic T cell (CTL) response plays a major role in viral control during spontaneous infection resolution. These cells develop an exhausted and pro-apoptotic status during chronic onset, being unable to get rid of HCV. The role of this response in contributing to sustained viral response (SVR) after anti-HCV is controversial. Recent studies show that after successful interferon-based anti-HCV treatment, HCV traces are still detectable and this correlates with a peak of HCV-specific CTL response activation, probably responsible for maintaining SVR by subsequent complete HCV clearing. Moreover, SVR patients' serum is still able to induce HCV infection in naïve chimpanzees, suggesting that the infection could be under the control of the immune system after a successful treatment, being transmissible in absence of this adaptive response. At least theoretically, treatment-induced viral load decrease could allow an effective HCV-specific CTL response reestablishment. This effect has been recently described with anti-HCV interferon-free regimes, based on direct-acting antivirals. Nevertheless, this is to some extent controversial with interferon-based therapies, due to the detrimental immunoregulatory α -interferon effect on T cells. Moreover, HCV-specific CTL response features during anti-HCV treatment could be a predictive factor of SVR that could have clinical implications in patient management. In this review, the recent knowledge about the role of HCV-specific CTL response in the development of SVR after anti-HCV treatment is discussed.

Key words: Hepatitis C virus; Chronic hepatitis; Hepatitis C virus-specific cytotoxic T cell response; Treatment; Direct-acting antivirals; Interferon-alpha; Ribavirin; Exhaustion; Apoptosis

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Core tip: Hepatitis C virus (HCV)-specific cytotoxic T lymphocyte (CTL) response plays an essential role in controlling acute HCV infection but its implication in treatment-induced viral control is controversial. During interferon/ribavirin treatment, HCV traces persist after sustained viral response (SVR) and this correlates with an activated HCV-specific CTL response, suggesting the necessity of this response to obtain an indefinite viral control. Current data propose that viral suppression during interferon/ribavirin treatment and during direct-acting anti-viral regimes could affect HCV-specific CTL restoration. Moreover, the features of this CTL response during treatment could have a predictive value on SVR outcome.

Larrubia JR, Moreno-Cubero E, Miquel J, Sanz-de-Villalobos E. Hepatitis C virus-specific cytotoxic T cell response restoration after treatment-induced hepatitis C virus control. *World J Gastroenterol* 2015; 21(12): 3480-3491 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3480.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3480>

INTRODUCTION

Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTLs) play an essential role in the natural control of HCV during acute infection^[1-4]. These cells are able to recognize HCV infected hepatocytes and destroy them, but they also secrete type I cytokines able to kill the virus in a non-cytopathic manner^[5-7] (Figure 1). Nevertheless, in a great percentage of cases the adaptive immune system is unable to get rid of HCV infection, consequently developing in the host a chronic infection^[8,9]. Chronic hepatitis C infection is featured by an impaired HCV-specific cytotoxic T cell response unable to control HCV replication^[5,10]. This response becomes exhausted in a first step due to the long-lasting high antigenemic burden^[11,12], featured by expression of negative co-stimulatory molecules and impaired proliferation and cytokine production^[13,14]. Finally, the continuous T cell stimulation could lead to the deletion of these cells by apoptosis induction^[15,16]. In these chronic cases, an anti-HCV treatment is compulsory to eliminate the infection, since the adaptive immune system is overwhelmed. Two main kinds of treatments can be currently offered to chronic hepatitis C patients; α -interferon-containing treatments and interferon-free regimes^[17]. In both cases, the treatment goal is to maintain an undetectable HCV-RNA by sensitive PCR six months after finishing treatment, which is considered a sustained viral response (SVR). α -Interferon combines an anti-viral effect plus immunomodulatory features^[18], while direct-acting antivirals (DAA) have a direct

action on HCV replication machinery^[19]. In both cases, these treatments could harness the immune system by releasing it from chronic antigenemia. This issue looks clear with DAA but it is more controversial with interferon-containing regimes due to the interferon suppressive effect on T cells^[20]. This point will be discussed in this review, along with whether it is necessary to restore an HCV-specific CTL response after anti-HCV treatment to obtain complete viral eradication from hidden sanctuaries in order to maintain an SVR, or on the other hand whether after successful treatment, these cells are not essential anymore because the infection is completely cleared.

RELATIONSHIP BETWEEN PERSISTENT VIRAEMIA AND IMPAIRED SPECIFIC CTL RESPONSE

HCV-specific CTL response is essential to control HCV infection but this becomes dysfunctional (exhausted) and can be deleted during chronic infection due to persistent antigenic stimulus^[11,12,15,16]. There are clear demonstrations of the inverse relationship between duration and level of viraemia and T cell responses in murine and human persistent viral infections^[21-23]. For instance, in hepatitis B virus infection there is an indirect correlation between viral load and specific-CTL response intensity^[24] and interestingly, viral load decrease by nucleos(t)ide analogues is able to restore an HBV-specific CTL response capable of exerting certain effector capacities^[25]. In chronic HCV infection, high viraemia leads to exhausted HCV-specific CTLs, featured by impaired proliferation, cytotoxicity and γ -interferon secretion^[26]. These cells are characterized by expression of negative co-stimulatory molecules such as PD-1^[11,13], down-regulation of the pro-survival IL-7 receptor^[15,27] and up-regulation of pro-apoptotic proteins, while this phenotype is not observed in cases with either infection resolution^[11,15], or those not targeting the infecting epitope owing to HCV escape mutations^[28]. These data suggest that viral load level could modulate the exhausted and pro-apoptotic phenotype on HCV-specific CTLs. Consequently, HCV burden decrease could help in HCV-specific CTL response restoration.

IS IT NECESSARY TO RESTORE THE HCV-SPECIFIC CTL RESPONSE TO OBTAIN A SUSTAINED VIRAL RESPONSE?

Although some studies suggest that HCV is completely eradicated from serum and peripheral blood mononuclear cells of spontaneously recovered or successfully treated patients^[29,30], this is a debated topic widely discussed in the literature. Reservoirs of HCV

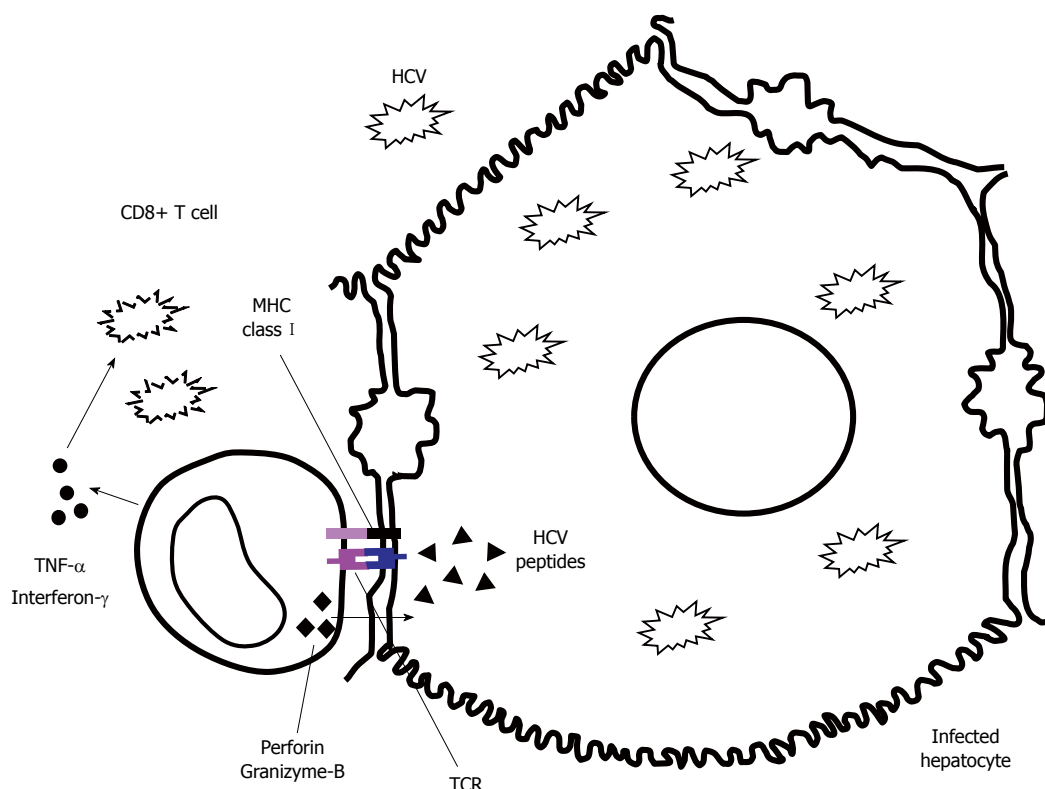


Figure 1 Hepatitis C virus-specific cytotoxic T lymphocyte effector abilities by either direct infected hepatocyte killing through release of perforin and granzyme-B or non-cytopathic hepatitis C virus deletion through γ -interferon and tumor necrosis factors- α secretion. HCV: Hepatitis C virus; TNF: Tumor necrosis factors.

infection could persist after a successful treatment, at least in interferon-containing regimes^[31-34]. In fact, negative and positive HCV RNA strands have been demonstrated in liver biopsies from SVR patients, indicating that HCV persisted and replicated in the livers of some sustained responders after treatment with α -interferon plus ribavirin^[34]. Other authors have also demonstrated in patients with SVR that small quantities of HCV RNA may persist in liver or macrophages and lymphocytes for up to 9 years after therapy. This continuous viral presence could result in persistence of humoral and cellular immunity for many years after therapy and could present a potential risk for infection reactivation^[32]. Moreover, in non-viraemic HCV antibody-positive patients, liver biopsies that are usually abnormal have been shown. Fibrosis was present in most cases with similar inflammatory infiltrate to viraemic cases. The presence of a CD8⁺ rich inflammatory infiltrate in those cases could suggest an ongoing immune response in the liver, supporting the view that HCV may persist in the liver in some of considered HCV-RNA-negative cases, analyzed with standard PCR techniques^[35]. Thus, all these data suggest that at least some SVR patients would not experience a complete HCV infection clearance, despite apparent clinical disease resolution, and in these cases HCV-specific CTL restoration could play an essential role in avoiding infection relapse. In fact, a longitudinal analysis of HCV RNA and HCV-

specific CTL response in SVR patients after treatment disclosed that HCV traces persisted several years after end of treatment and interestingly, these HCV RNA peaks matched with a detectable HCV-specific CTL response, expressing activation markers and, suggesting that these cells could keep the virus under control after SVR^[36]. Additionally, the sera of these SVR patients could also be infective, since chimpanzees have been infected, using the serum from resolver cases with detectable HCV traces after complete clinical infection resolution due to anti-HCV treatment^[37]. It is true that very few cases of HCV reactivation after infection resolution have been described^[38,39], in contrast to what happens in HBV infection, mainly in immunosuppressed patients^[40]. In fact, during clinical practice, most of the HCV patients who show sustained undetectable viraemia with usual PCR assay develop a permanent clinical control, while in HBV infection is more common the viral reactivation under immune-suppressive conditions. Nevertheless, this does not mandatory mean that after SVR, HCV is completely cleared by interferon/ribavirin treatment. In fact, the data previously summarized show that HCV replication a long period after SVR development can be demonstrated but probably, these viral traces are finally completely deleted by the HCV-specific-CTL response, avoiding thus far the possibility of a late HCV infection reactivation. Thus, checking the quality of the HCV-specific CTL-response would be useful for tracking

those patients who develop immunosuppression soon after developing SVR and consequently, could be at risk of recurrence until their immune system is restored^[38,39]. On the other hand, in interferon-free regimes, based on DAA combination, there is not yet any data about occult infection after SVR. These therapeutic combinations are highly effective in controlling the infection but what has not been addressed yet is whether adaptive immune response collaboration is necessary to obtain the desired goal. Before treatment, there are naturally occurring resistant variants to DAA combinations^[41-43], because of the high mutation rate of HCV during replication, due to the lack of proofreading function in the HCV RNA polymerase^[44]. It is true that viral resistance to DAA regimens will occur in a reduced number of virions, but at least statistically, this situation could happen and, in this setting it could be necessary to combine the effect of these powerful drugs with the immune system action to get rid of the minor resistant variants to DAA regimes.

INTERFERON-BASED TREATMENTS

α -Interferon can have a direct antiviral activity by induction of interferon stimulated genes, such as protein kinase R (PKR)^[45], oligoadenylate synthetase (OAS)^[46], myxovirus resistance protein (Mx)^[47], apolipoprotein B mRNA editing enzyme-catalytic polypeptide-like (APOBEC)^[48,49] or tripartite motifs (TRIM), which directly inhibit viral replication^[18]. Moreover, α -interferon develops an indirect anti-viral effect by affecting the innate and adaptive immune response through expansion of activated specific CD8⁺ T cells^[50-52] and NK cells^[52,53], up-regulation of proteins of the antigen presentation machinery^[54], maturation of dendritic cells (DC)^[55], and augmentation of B-cell responses^[51,56]. With respect to adaptive cytotoxic response, α -interferon could boost specific-CTL response by expansion of activated cells^[50,57] but also by blocking exhausted status by antigen burden decrease^[58,59]. Nevertheless, α -interferon can also have a negative effect on T cell numbers due to regulation of T cell recirculation^[20]. The dynamics of HCV viral load during PEG- α -interferon/ribavirin treatment has two phases. First decay is rapid and it is assumed to be owing to PEG- α -interferon direct effect, while second phase decay is slower and it is probably caused by either adaptive immune response^[60] or NK cell activation^[61,62]. However, NK cells very soon after starting treatment are already activated and, therefore they could act during the first phase decay. Actually, according to mathematical methods, the second decay of HCV viral kinetics during treatment is due to the effect of cellular immune response, mainly specific-CTLs and NK cells^[63-65]. NK cells constitute an early host defense against viral pathogens^[66,67], eliminating virus-infected cells both directly through cytolytic mechanisms and indirectly by secreting cytokines such

as γ -IFN^[68]. Although NK cells have been classically viewed as innate immune cells, their effects could extend into periods of adaptive immunity, and hepatic NK cells also demonstrate adaptive immunity to structurally diverse antigens^[69,70]. NK cell frequency increases as early as hours following the initiation of antiviral therapy and this is associated with early viral response^[61,62], which could contribute to HCV-specific CTL restoration by decreasing initially the viral burden. Interestingly, absence of a strong HCV viral load decrease at week 12 of PEG- α -interferon/ribavirin treatment has 100% negative predictive value of SVR and it is used as a treatment stopping rule^[71]. This effect could be related to the impairment of HCV-specific immune response at that point. However, the role of PEG- α -interferon/ribavirin treatment on HCV-specific T cell kinetics has not been completely addressed *in-vivo* and, it is a controversial issue. In the early phase of infection, α -interferon-based treatment can rescue a polyfunctional long lived HCV-specific CTL response, while this issue is not clear in long-lasting infection^[72,73]. A previous paper correlated the development of SVR after PEG- α -interferon/ribavirin treatment with induction of a HCV-multispecific CD4⁺ Th-1 response^[74]. Another study suggested that antiviral therapy-induced viral clearance may be associated with the induction, expansion, and/or recirculation of HCV antigen-specific cytolytic T cells, and it may play a role in the maintenance of a non-viraemic state^[75]. Moreover, a longitudinal improvement in NS3-specific CTL response during interferon-based treatment has also been described^[76]. Furthermore, a core-specific and NS3-specific CTL response restoration in SVR patients featured by higher frequency and cytotoxicity after α -interferon-based treatment has also been reported^[77]. In that work, a peak of specific-CTL response in SVR patients between treatment week 4 and week 12 was observed. A similar finding was stated by Tatsumi *et al.*^[78], describing an NS3-specific and core-specific CTL response increase in SVR patients after four weeks of treatment with α -interferon/ribavirin treatment. Nevertheless, another work focused on CD8 and CD4 specific responses did not show a clear association between T cell reactivity and treatment outcome although they found an enhancement of proliferative T-cell responses during therapy^[79]. Additionally, a recent retrospective analysis of peripheral blood lymphocyte samples from DITTO study^[80] likewise did not show a correlation between restoration of HCV-specific CTL response and SVR after PEG- α -interferon/ribavirin treatment although patients presenting a better HCV-specific CD8 cell proliferative potential at baseline were more likely to present a rapid and sustained viral response^[81]. Curiously, a work carried out by our group observed a similar treatment outcome independently of the frequency of peripheral cells at base line. Nevertheless, an improvement of peripheral HCV-specific CTL frequency after 12 wk of treatment correlated with SVR development^[60] (Figure

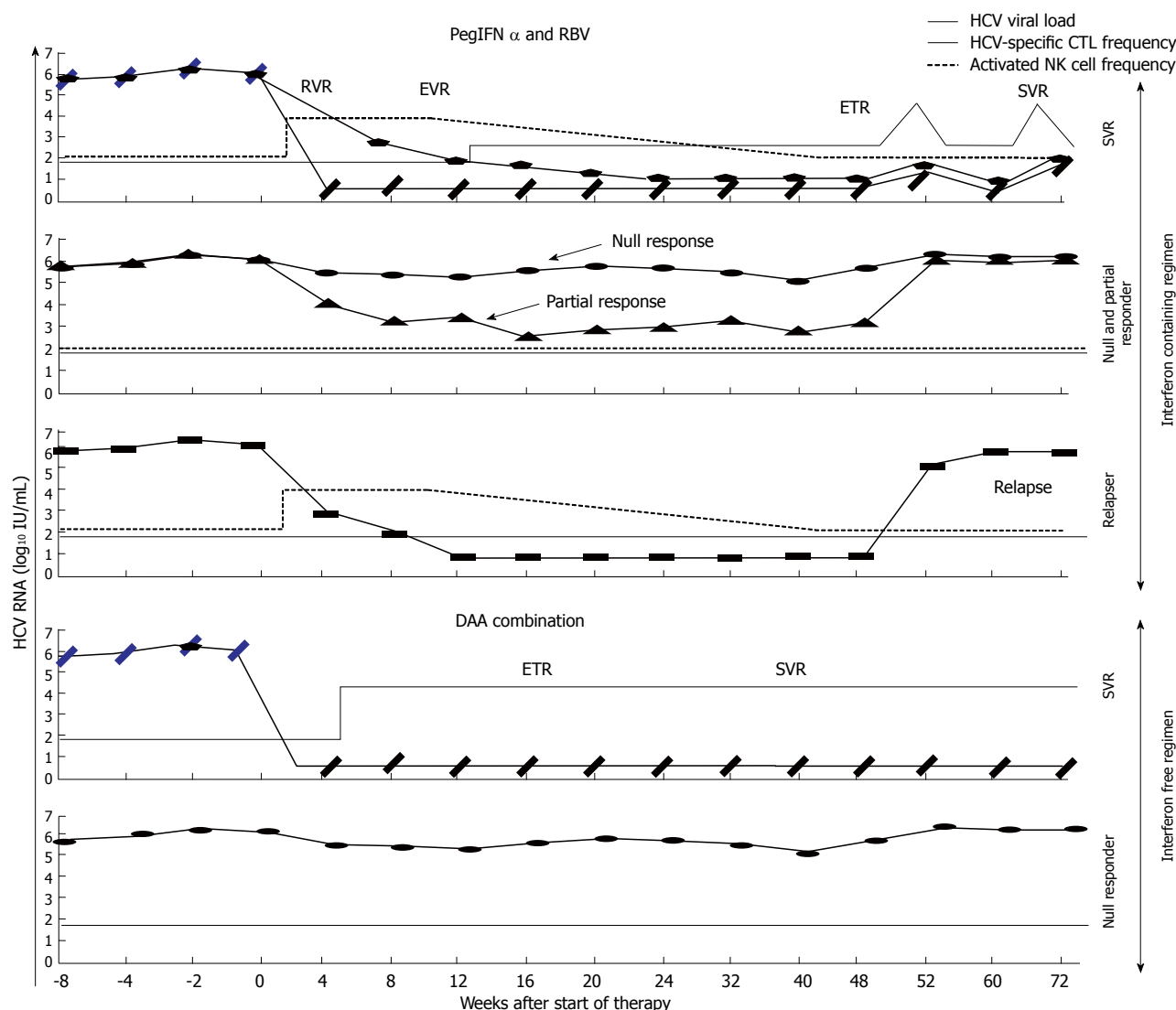


Figure 2 Theoretical hepatitis C virus viral load, hepatitis C virus-specific cytotoxic T lymphocyte frequency and activated natural Killer cell frequency kinetics during anti-hepatitis C virus treatment with interferon-free and interferon-containing regimens, according to the different types of response. PegIFN: Pegylated- α 2-interferon; RBV: Ribavirin; DAA: Direct acting antiviral; NK: Natural Killer cell; CTL: Cytotoxic T lymphocyte; HCV: Hepatitis C virus; RVR: Rapid viral response; EVR: Early viral response; ETR: End of treatment response; SVR: Sustained viral response.

2), in concordance with Tatsumi *et al.*^[78] and Caetano *et al.*^[77] data. Therefore, treatment could be able to restore HCV-specific CTL numbers, at least in one subpopulation of patients. This finding was not described in Pilli's work^[81] but was suggested in Barnes' study^[79] and it could be due to the type of samples analyzed. In our research^[60], directly *ex-vivo* cells were checked, while in Pilli's paper^[81] frozen cells were retrospectively tested and the thawing process could have affected the more exhausted cells, preventing their detection thus far. On the other hand, investigations analyzing HCV-specific T cell response by γ -interferon-ELISPOT do not support that treatment-induced viral clearance is associated with an enhanced antiviral T cell response^[82,83]. The apparent discrepancy between these observations could be explained by the manner in which specific T cells are quantified, since in former studies, cells were recognized by their ability to

produce type-I cytokines and, in Larrubia's work, those cells were visualized directly by HLA-I/epitope multimeric complex labelling. Consequently, the HCV-specific CTL number restoration observed in a subpopulation of infected cases in Larrubia's study would not be able to secrete γ -interferon yet^[15], as a result those cells would not be detected by Barnes *et al.*^[79]. Thus, the functionality of these cells should be further analyzed since they could be present but dysfunctional, although a specific proliferative potential improvement during treatment was observed, suggesting a certain degree of functional restoration. Besides, a decrease of programmed cell death protein (PD-1) expression on HCV-specific CTLs after treatment in SVR patients was also described (Figure 3). PD-1 is an immunoreceptor tyrosine-based inhibition motif-containing (ITIM-containing) expressed on activated T cells that mediates hyporesponsiveness^[84]. PD-1

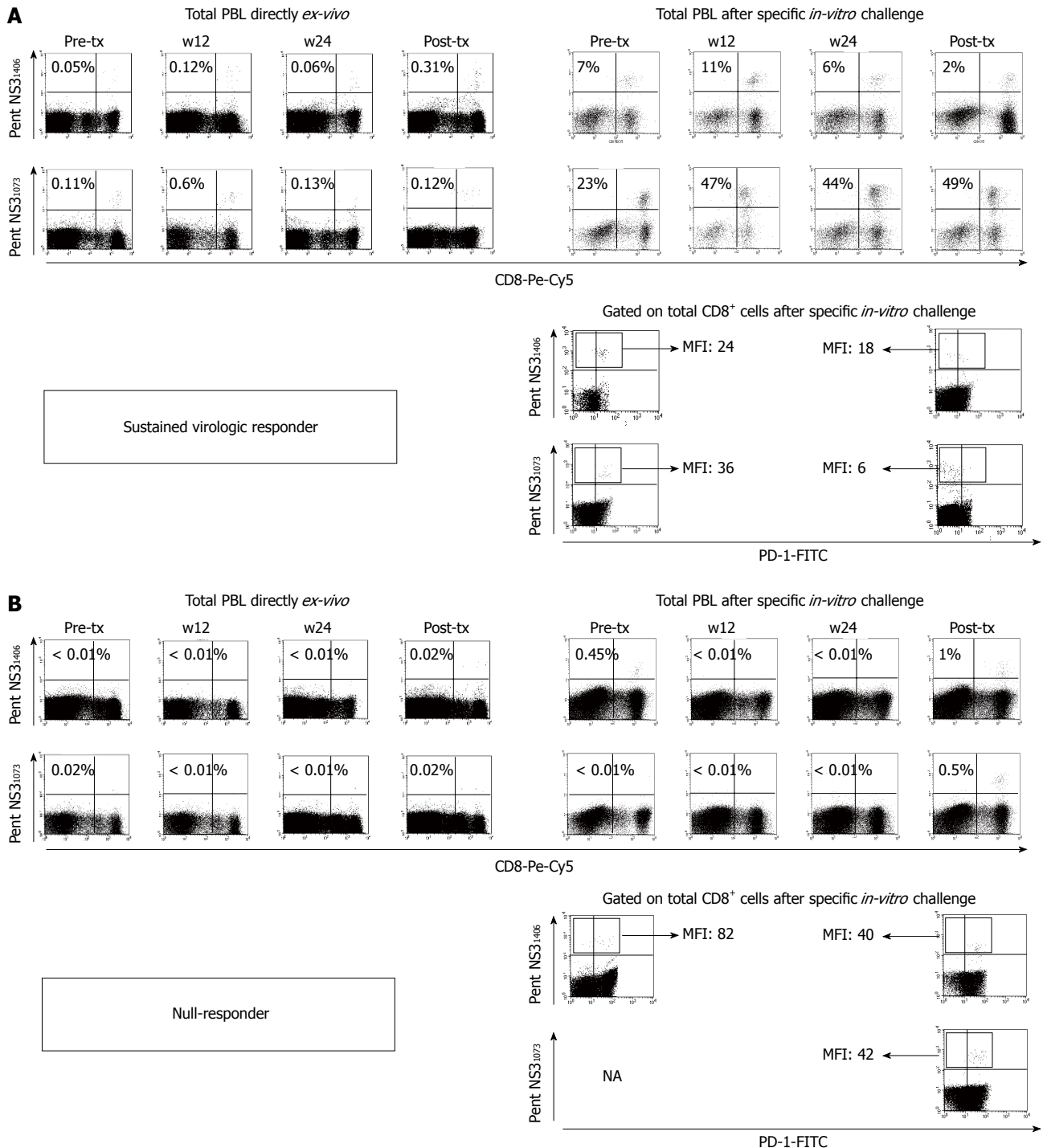


Figure 3 Representative FACS® dot-plots of peripheral blood mononuclear cells from a sustained viral responder (A) and a null responder (B) after treatment with pegylated- α 2-interferon plus ribavirin. The dot-plots show the frequency of hepatitis C virus (HCV)-specific cytotoxic T lymphocyte (CTLs) directly *ex-vivo* and after specific *in-vitro* challenge, besides the PD-1 phenotype pre- and after treatment. PBL: Peripheral blood lymphocytes; MFI: Mean fluorescence intensity; tx: Treatment; NA: Not available; PentNS3₁₄₀₆: Multimeric complexes HLA-A2/NS3₁₄₀₆ phycoerythrin labelled; PentNS3₁₀₇₃: Multimeric complexes HLA-A2/NS3₁₀₇₃ phycoerythrin labelled.

expression on HCV-specific CTLs inversely correlates with early and sustained virologic response to IFN-based antiviral therapy^[85] and, it is associated with impaired control of *in-vitro* replication^[86]. Therefore, a certain degree of HCV-specific CTL function restoration after treatment in our work was also observed, since the negative co-stimulatory molecule PD-1 was down-

regulated in treatment responders^[60]. In this work, only patients with a high viral load decrease during the first 12 wk of treatment had detectable HCV-specific CTL at week 12 and this was maintained throughout the treatment (Figure 3). A similar correlation between viral load and T cell response kinetics in other chronic hepatotropic non-cytopathic viral infections has already

been shown^[24]. Taken together, these data suggest that in chronic hepatitis C cases with conserved HCV-specific T cells, a rapid viral load decrease could suppress the exhausted status on those cells, allowing their peripheral detection. Nevertheless, in patients with either deleted HCV-specific CTLs or with low HCV viral load decrease due to interferon insensitivity it would be impossible to restore a detectable HCV-specific CTL response. As a result, it could be possible to speculate that although type- I interferon associates with lymphopenia induction that could impair specific-T cell number by affecting lymphocyte re-circulation^[20], it also allows for an increase in frequency of those cells by favoring clonal expansion and memory formation^[87] and by decreasing T cell exhaustion through viral load reduction^[24]. The balance between those α -interferon effects would be responsible for the peripheral detection of these cells during treatment, although these cells will still show an exhausted status that will disappear after developing an SVR. Additionally, during interferon based treatment, NK cells have a major role in controlling infection^[61,62] and this will help in HCV-specific CTL response restoration after treatment^[60,75-78], which will finally contribute to indefinite HCV control and eradication^[36]. Consequently, the current information published suggests that in cases with SVR an HCV-specific CTL response improvement during treatment can be observed, although it is still dysfunctional but acquires effector capacities after end of treatment and is probably responsible for destroying viral traces (Figure 3). Unfortunately, there are not data yet about HCV-specific CTL response during treatments combining α -interferon with the new DAA^[88], but we already have some information about DAA combination regimes and this will be discussed in the following lines.

INTERFERON FREE REGIMES

Direct-acting antivirals are able to eliminate infection through affecting the function of different HCV proteins involved in HCV replication, such as NS3/NS4 protease, NS5A complex and NS5B polymerase^[17]. Interestingly, DAA combinations could get rid of HCV infection by preventing the effect of naturally occurring resistant variants against one or another single DAA^[41-43]. However, it is still not clear whether this treatment is sufficient to eliminate HCV infection or if it is also necessary to restore an efficient HCV-specific CTL response to eradicate the viral traces from specific sanctuaries and to eliminate potential resistant variants to DAA combinations. Recent work has analyzed the role of HCV-specific CTL response in chronic HCV patients treated with interferon-free therapy^[89]. In this paper is nicely shown that responder patients to DAA therapy recover a HCV-specific CTL response with the ability to proliferate after antigen encounter, while this is not observed in non-responder cases (Figure 2). These findings are similar to the ones reported

in some studies with IFN-based regimens^[60,74-78], but more intense probably because in both cases a positive effect of viral load decrease on HCV-specific T cell is present but, in DAA regimens the predominantly negative IFN immune suppressive influence on T cells is avoided^[90]. Moreover, this work not only shows an increase in the number of T cells but it also describes an improvement in the quality of the response by down-regulation of negative co-stimulatory molecules and by restoring its cytolytic activity. Thus, this finding supports a role for DAA viral clearance in driving recovery of HCV-specific CTLs but we do not still know if this restoration play a role in controlling HCV infection during treatment or it is only an epiphenomenon. The same consistent adaptive T cell response restoration has been described in chronic HBV infection during nucleos(t)ide analogue treatment, reinforcing these data the idea that, at least in cases without specific-T cell deletion, intense viral load decrease can restore a previously exhausted T cell response^[25,91]. According to this observation, it is possible to speculate that specific-CTL restoration during DAA regimes could contribute to the high-sustained cure rates and the low relapse frequency described with this kind of therapies. In Table 1, the studies in favor or against HCV-specific CTL role in SVR after interferon-free and -containing regimes are summarized.

RESTORATION OF A HCV-SPECIFIC CTL RESPONSE AS PREDICTIVE FACTOR OF SVR

According to the previous data, the extent of T cell reconstitution might be able to be used as a guide to personalize anti-HCV therapy and as a predictive factor of response. A lack of induction of T cells could signal the need for therapy intensification and conversely, a good T cell recovery could allow shorter treatment duration in some patients, with the expectation that any residual virus would be cleared by T cells. These issues have not been completely addressed yet, but there are a few data that will be discussed in the next lines. To address whether restoration of peripheral HCV-specific CTL number could correlate with SVR rate and behave as a treatment response prognosis factor, a multivariate analysis was carried out by our group during interferon-based treatment^[60]. In that analysis, the increase of detectable HCV-specific CTLs after 12 wk of treatment was an independent factor related with SVR. Moreover, the predictive value of this variable on early or delayed viral responder (EDVR)^[71] genotype-1 cases was also analyzed to understand its role in a hypothetical guided-therapy decision process. Interestingly, all the EDVR genotype-1 samples with detectable HCV-specific CTLs at w12 developed SVR, which means a 100% positive predictive value (PPV). This data could be useful to encourage these patients to finish treatment, but it could also be a decision rule

Table 1 Summary of published works about hepatitis C virus-specific cytotoxic T lymphocyte during anti-hepatitis C virus treatment pointing-out the data in favor and against hepatitis C virus-specific cytotoxic T lymphocyte response restoration either during or after treatment

Ref.	Year	Treatment	Samples	CTL visualization	In favor	Against
Martin <i>et al</i> ^[89]	2014	DAA	Frozen PBMC	Tetramer staining	Restoration of HCV-specific CTL response during treatment in SVR cases	
Larrubia <i>et al</i> ^[60]	2013	PEG-IFN/ RBV	Fresh PBMC	Pentamer staining	HCV-CTLs detection at week 12 of treatment correlates with infection resolution Detection of PD-1 ^{low} HCV-specific CTLs after treatment correlates with SVR	
Humphreys <i>et al</i> ^[82]	2012	PEG-IFN/ RBV	Frozen PBMC	γ -IFN ELISPOT assay		Treatment induced genotype-3a viral clearance is not associated with an enhancement of antiviral T cell response
Tatsumi <i>et al</i> ^[78]	2011	PEG-IFN/ RBV	Fresh PBMC	γ -IFN ELISPOT assay	HCV-specific CTL frequency increase between baseline and treatment week 4 was observed in SVR but not in non SVR patients	
Barnes <i>et al</i> ^[83]	2009	PEG-IFN/ RBV	Frozen PBMC	γ -IFN and IL-2 ELISPOT assay		Not enhanced specific T cell response during high dose IFNa therapy was reported
Caetano <i>et al</i> ^[77]	2008	PEG-IFN/ RBV	Fresh PBMC	Pentamer staining	Chronically infected patients who responded to treatment showed a higher HCV-specific CTL frequency than non-responders during and post-treatment Terminally differentiated effector cells increased more rapidly in responders, and their frequency was always higher than in non-responder patients Patients with sustained viral response showed a decrease in PD-1 expression on HCV-specific CTLs after therapy completion	
Golden-Mason <i>et al</i> ^[85]	2008	PEG-IFN/ RBV	PBMC	Pentamer staining	Early therapeutic intervention during the acute phase of HCV infection reconstituted a long-lived polyfunctional memory T cell response	
Badr <i>et al</i> ^[73]	2008	PEG-IFN	Frozen PBMC	Tetramer staining		
Pilli <i>et al</i> ^[81]	2007	PEG-IFN/ RBV	Frozen PBMC	γ IFN ELISPOT assay and tetramer staining		No significant correlation between HCV-specific CTL kinetics and viral decay during treatment
Morishima <i>et al</i> ^[75]	2003	IFN/RBV	Fresh PBMC	Chromium-51 release assay	Subjects who had completed the treatment and had undetectable HCV RNA levels after therapy had a detectable HCV-specific CTL response more frequently than those who were viraemic	
Vertuani <i>et al</i> ^[76]	2002	IFN	Fresh PBMC	Chromium-51 release assay	Increase in the intensity of HCV epitope recognition by HCV-specific CTL after 1 to 5 m of treatment	
Barnes <i>et al</i> ^[79]	2002	IFN/RBV	Frozen PBMC	γ IFN ELISPOT assay		T cell responses induced during high dose of IFNa treatment appear not to influence the virological outcome

DAA: Direct acting anti-viral; IFN: Interferon- α 2; RBV: Ribavirin; PBMC: Peripheral blood mononuclear cells; γ -IFN: Gamma-interferon; SVR: Sustained viral response.

to maintain double therapy in cases with positive cells or to add a DAA in patients without detectable HCV-specific CTLs at w12, which could have interest from an economical point of view in countries restricting the free use of DAA^[92,93]. Consequently, the excellent PPV treatment response of HCV-specific CTL detection at w12 could be complementary to the high negative predictive value of a viral load decrease lower than 2log at w12 of PEG- α -interferon/ribavirin treatment, as a strategic tool in the clinical decision process during treatment. We should await similar studies on DAA-based regimes to verify whether HCV-specific

CTL restoration during treatment could be used as a response predictive factor and as a tool to guide therapy.

CONCLUSION

Treatment-induced viral load decrease can restore a reactive HCV-specific CTL response during treatment in DAA-based treatments and this can influence the high rate of sustained response observed with this regime. In IFN-based treatment, HCV-specific CTL restoration in some cases can be observed and this

issue has an excellent positive predictive value in developing an SVR outcome. In this setting, NK cells develop a major role in viral load decrease that could impact on HCV-specific CTL restoration, although still displaying exhausted features. However, this response reaches a non-exhausted phenotype after treatment in SVR cases and it correlates thereafter with activation peaks coinciding with HCV detection after HCV clinical resolution, suggesting an important role in maintaining SVR and in the complete HCV eradication after treatment. Therefore, taking into account all these data, we can suggest that a potent anti-viral treatment could not be enough alone to obtain a high cure rate, but probably it is also necessary to restore a powerful HCV-CTL response to get that goal.

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Long-chain acyl-CoA synthetase in fatty acid metabolism involved in liver and other diseases: An update

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Abstract

Long-chain acyl-CoA synthetase (ACSL) family members include five different ACSL isoforms, each encoded by a separate gene and have multiple spliced variants. ACSLs on endoplasmic reticulum and mitochondrial outer membrane catalyze fatty acids with chain lengths from 12 to 20 carbon atoms to form acyl-CoAs, which are lipid metabolic intermediates and involved in fatty acid metabolism, membrane modifications and various physiological processes. Gain- or loss-of-function studies have shown that the expression of individual ACSL isoforms can alter the distribution and amount of intracellular fatty acids. Changes in the types and amounts of fatty acids, in turn, can alter the expression of intracellular ACSLs. ACSL family members affect not only the proliferation of normal cells, but the proliferation of malignant tumor cells. They also regulate cell apoptosis through different signaling pathways and molecular mechanisms. ACSL members have individual functions in fatty acid metabolism in different types of cells depending on substrate preferences, subcellular location and tissue specificity, thus contributing to liver diseases and metabolic diseases, such as fatty liver disease, obesity, atherosclerosis and diabetes. They are also linked to neurological disorders and other diseases. However, the mechanisms are unclear. This review addresses new findings in the classification and properties of ACSLs and the fatty acid metabolism-associated effects of ACSLs in diseases.

Key words: Long-chain acyl-CoA synthetase; Fatty acid; Proliferation; Apoptosis; Liver diseases; Metabolic diseases; Pathways

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Core tip: Recent research has shown that long-chain acyl-CoA synthetase (ACSL) family members

have individual functions in fatty acid metabolism in different types of cells depending on substrate preferences, subcellular location and tissue specificity, thus contributing to several diseases. These enzymes also regulate cell proliferation and apoptosis through different mechanisms. This review addresses new findings in the fatty acid metabolism-associated effects of ACSLs in diseases.

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INTRODUCTION

Fatty acids are a major source of energy in mammals. Given a sufficient oxygen supply, fatty acids can be degraded to CO₂ and H₂O in the body and large quantities of energy are released in the form of adenosine triphosphate (ATP), which can then be used by the organism. Exogenous or endogenous fatty acids are chemically quite inert and need to be activated to form acyl-CoA in the cell, outside the mitochondrion, before they enter a metabolic pathway. Acyl-CoA synthetase on the endoplasmic reticulum (ER) and mitochondrial outer membrane catalyzes the conversion of fatty acids to acyl-CoA in the presence of ATP, CoA and Mg²⁺.

The acyl-CoA synthases can be classified, according to the carbon chain length of the fatty acid they catalyze, into very long-chain acyl-CoA synthases (ACSVL), long-chain acyl-CoA synthases (ACSL), medium-chain acyl-CoA synthases (ACSM) and short-chain acyl-CoA synthases (ACSS). ACSLs mainly catalyze fatty acids with chain lengths of 12-20 carbons^[1]. ACSL1 was initially reported in 1953 and ACSL3 to 6 were subsequently discovered in succession^[2]. There are five different ACSL isoenzymes in mammals, and each isoenzyme has multiple spliced variants. Mouse ACSL4 variants 1 and 3 are equivalent to the human variants 2 and 1, respectively^[1]. ACSL1, ACSL5 and ACSL6 (formerly ACSL2) show a 60% amino acid sequence homology. ACSL3 and ACSL4 show a 68% homology with each other^[3,4]. There are many differences in the five ACSLs with regard to regulating fatty acid metabolism, promoting cell proliferation and apoptosis and causing diseases.

EFFECTS OF ACSL ON FATTY ACID METABOLISM

ACSLs are characterized by substrate and tissue specificity. The expression of ACSL family members

varies in different tissues and in its subcellular location. For example, ACSL6 has been reported to be mainly expressed in neural cells and the brain, and ACSL3 protein is predominant in brain and testis tissue. However, it is undetectable in the heart^[5,6]. ACSL5 is localized in the outer mitochondrial membrane and microsomes^[7].

ACSL1

ACSL1 is the predominant isoform in the liver. The activity of ACSL1 accounts for 50% of total hepatic ACSL activity^[8]. ACSL1 over-expression increases the proportion of oleic acid in diacylglycerol (DAG) and phospholipids (PLs), but reduces the amount of oleic acid in cholesterol^[9]. ACSL1 and ACSL5 are thought to play an important role in partitioning fatty acids toward triglyceride (TG) synthesis^[10]. Deficiency of ACSL1 causes reduced synthesis of TG, but facilitates β -oxidation and the synthesis of PL species in hepatic cells^[8]. Over-expression of ACSL1 results in the generation of large amounts of TG and its accumulation in hepatoma cells^[11]. ACSL1 is able to activate membrane transport to effectively import free fatty acids into cells^[12]. Compared with the normal control group, ACSL1 protein expression was observed to increase 2.468-5.418-fold 48 h after hepatocytes were treated with various concentrations of free long-chain fatty acids. In the presence of excessive free fatty acids, hepatocytes increase their uptake of free fatty acids and deposit them in the form of TG^[13].

ACSL3

ACSL3 is mainly expressed during fetal development, and the level of ACSL3 in adult cerebrum is only 10% of its maximum on 15 d after birth^[14]. ACSL3 is localized in the ER and cytosolic lipid droplets (LDs)^[15]. LDs are the main organelles for the storage of neutral lipids, and contribute to the maintenance of lipid homeostasis. The physiological function of ACSL3 is to promote the synthesis of lecithin and the formation of LDs, which is not seen in other isoforms of ACSL^[16]. Lecithin is the main PL on the surface of very low density lipoprotein (VLDL). Secretion of VLDL is suppressed in case of ACSL3 insufficiency. ACSL3 has an influence on the secretion of VLDL through the promotion of lecithin synthesis provided by the ACSL3-mediated synthesis of DAG^[17]. The N-terminal region of ACSL3 is required for fatty acid uptake, due to its effect on enzymatic activity^[18]. Experimental findings have shown that ACSL3 is able to regulate lipogenesis by facilitating the gene activity of several lipogenic transcription factors, including peroxisome proliferator-activated receptor- γ (PPAR- γ), carbohydrate-responsive element-binding protein (ChREB) and sterol regulatory element-binding protein-1c (SREBP-1c)^[17,19,20].

ACSL4

ACSL4 is mainly expressed in adrenal glands and

other steroid producing organs. ACSL4 expression is related to steroid hormones and growth factor receptors^[21]. Silencing ACSL4 can inhibit the generation of hormone-related steroid^[22]. ACSL4 has a substrate preference for arachidonic acid (AA). The catalytic activity of ACSL4 for AA is 5 to 6 times higher than that for linoleic acid^[4]. ACSL4 expression decreases the conversion of free AA into leukotrienes while increasing the conversion of free AA into prostaglandins^[21,23]. ACSL4 over-expression also significantly increases the synthesis of eicosanoid-CoA and promotes conversion of AA into phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and TG^[23,24].

ACSL5

It is well known that the absorption of dietary long chain fatty acids largely occurs in the jejunum and ACSL5 is mainly expressed in the small intestine. This suggests that ACSL5 plays a crucial role in the absorption of dietary long-chain fatty acids. Studies on ACSL5 have shown that it can catalyze the metabolism of exogenous, but not *de novo* synthesized fatty acids^[7,25]. ACSL5-knockout mice did not show changes in the absorption of long-chain fatty acids or weight gain after a high-fat diet^[26]. ACSL5 is also expressed in the liver and brown adipose tissue characterized by high levels of TG synthetase, and is involved in the synthesis of TG. ACSL5 over-expression promotes the synthesis of DAG and TG from fatty acids, which may be attributed to accelerated re-acylation by ACSL5. In contrast, ACSL5-knockout resulted in decreased synthesis of TG^[11,27]. In addition, ACSL5 is also the only ACSL isoform localized in mitochondria. It is commonly believed to be related to β -oxidation. However, some data have indicated that ACSL5 over-expression does not have an impact on the β -oxidation of fatty acids^[7].

ACSL6

ACSL6 also catalyzes very long-chain fatty acids (C18 to C26) to form acyl-CoAs. Following the supplementation of polyunsaturated fatty acids to cells over-expressing ACSL6, both polyunsaturated fatty acids and saturated fatty acids increased in the cells, but to different degrees. Compared with oleic acid and AA, over-expression of ACSL6 in PC12 cells preferentially promotes docosahexaenoic acid (DHA) to form DHA-CoA and to further synthesize PLs and TGs. ACSL6 over-expression increases the level of PLs, but does not alter the distribution of fatty acids among the major PL species^[28].

EFFECTS OF ACSL ON CELL PROLIFERATION

ACSLs affect the proliferation of normal cells. ACSL5 contributes to cell proliferation along the intestinal crypt-villus axis (CVA)^[29]. Schoonjans *et al.*^[30] induced

liver cell proliferation through partial liver resection, and ACSL mRNAs in the liver cells almost completely disappeared within 24 h after the surgery; then restored to 40% of the preoperative level 48 h later; and to 70% of the preoperative level 72 h later. These results suggest a substantial connection between ACSL and the proliferation of normal cells.

ACSL is also associated with the proliferation of malignant tumor cells. Tumor cells may over-express ACSL to utilize fatty acids as an energy source for cell proliferation. Studies have shown that ACSL4 expression is induced in MCF-7 and SKBr3 breast cancer cells and this promotes the proliferation of tumor cells^[21,31]. In addition, ACSL4 is also involved in cell proliferation in liver cancer and colon cancer^[32,33]. ACSL6 was found to be related to tumor cell proliferation in experiments on neuroblastoma cells and pheochromocytoma.

EFFECTS OF ACSL ON CELL APOPTOSIS

ACSLs not only affect cell proliferation, but also play a role in cell apoptosis. Studies have shown that ACSL6 expression is elevated in animal models of non-alcoholic fatty liver disease (NAFLD), which may promote liver cell apoptosis^[34]. In apoptotic intestinal cells, ACSL5 down-regulates cellular Fas associated death domain-like interleukin-1 β converting enzyme inhibitory protein (cFLIP), which has been proven to be anti-apoptotic, and up-regulates tumor necrosis factor-related apoptosis inducing ligand receptor 1 (TRAIL-R1), which is the membrane receptor of the tumor necrosis factor/c-Jun N-terminal kinase (TNF/JNK) apoptosis pathway. A study has also shown that ACSL5 induces synthesis of ceramide, an important second messenger of the apoptosis pathway^[35] (Figure 1). A study on an animal model of systemic lupus erythematosus showed that transcription of ACSL5 was significantly elevated in the disease model compared with normal controls and silencing ACSL5 with siRNA reduced apoptosis^[36].

Different fatty acids have different effects on cell apoptosis. Saturated fatty acids such as palmitic acid and stearic acid can activate caspase 3 (Figure 1), which leads to cell apoptosis, while unsaturated fatty acids have little effect on cell apoptosis. Studies have shown that ACSL1 over-expression can further enhance palmitoyl lipid-induced apoptosis, while treatment with triacsin C, an ACSL inhibitor, reverses the pro-apoptotic effect of saturated fatty acids^[37].

ACSL AND LIVER DISEASES

With the exception of ACSL6, all ACSL isoforms are expressed in the liver and the major subtype of ACSL in the liver is ACSL1, which is also a target gene of peroxisome proliferator-activated receptor- α (PPAR- α). PPAR- α is involved in fatty acid metabolism^[38].

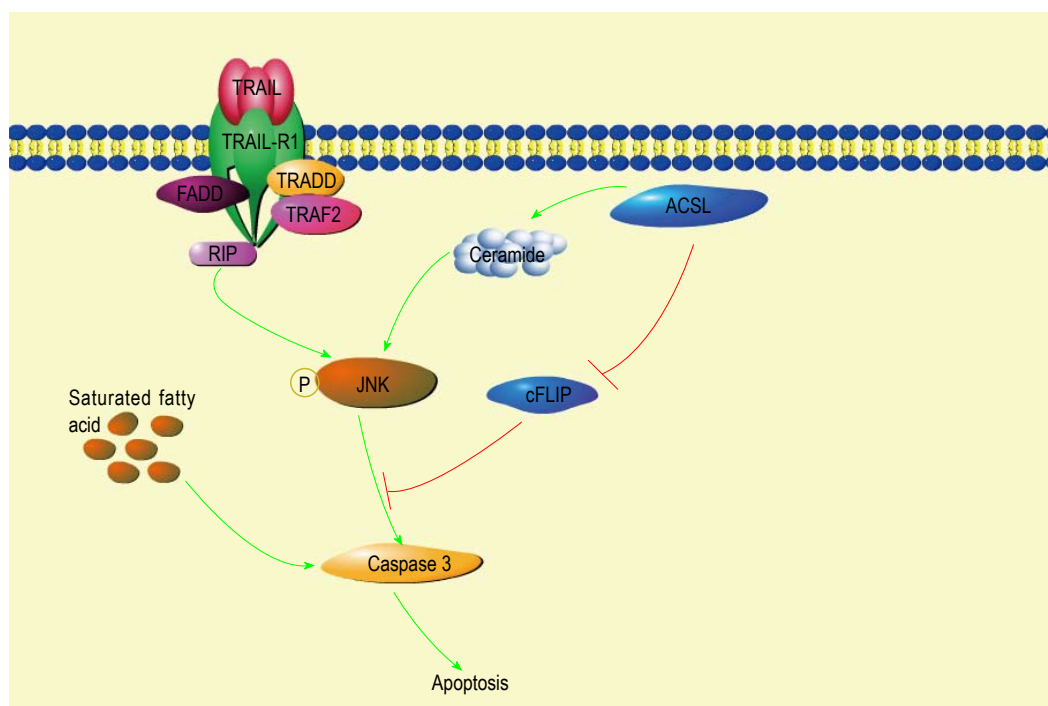


Figure 1 Long-chain acyl-CoA synthetases induce apoptotic cell death via the c-Jun N-terminal kinase pathway. JNK can be activated via phosphorylation by increased ceramide which can be induced by ACSLs that subsequently activate caspase 3, leading to apoptotic cell death. This shows that ACSLs may induce apoptotic cell death via the JNK pathway. In addition, anti-apoptotic proteins such as cFLIP, which is downregulated by ACSLs, may inhibit the activation of JNK. ACSL: Long-chain acyl-CoA synthetase; cFLIP: Cellular Fas associated death domain-like interleukin-1 β converting enzyme inhibitory protein; JNK: c-Jun N-terminal kinase; TRAIL: Tumor necrosis factor-related apoptosis inducing ligand; TRAIL-R1: Tumor necrosis factor-related apoptosis inducing ligand receptor 1.

Deficient or repressed PPAR- α expression in the liver leads to decreased activity of ACSL1 and some other key enzymes involved in fatty acid metabolism, and consequently fat deposition and inflammation which promote liver fibrosis^[39]. Another subtype of ACSL, ACSL3, plays an important role in the pathogenesis of fatty liver in addition to ACSL5^[25,40]. Silencing ACSL3 inhibits the release of HCV particles from infected liver cells into plasma. HCV proliferation in liver cells can cause structural and functional changes or interfere with protein synthesis, and ultimately degeneration and necrosis of liver cells^[17]. ACSL4 is upregulated in patients with NAFLD who have undergone bariatric surgery^[41]. The regulation of ACSL4 may be through both the 3'-5'-cyclic adenosine monophosphate (cAMP) and p38 mitogen-activated protein kinase (MAPK) pathways in liver cancer^[32] (Table 1).

ACSL AND METABOLIC DISEASES

Fatty acid metabolism disorder is involved in the occurrence of many metabolic diseases, such as obesity, diabetes, cardiovascular disease and atherosclerosis. ACSLs play an important role in fatty acid metabolism, and dysfunction of these enzymes often leads to fatty acid metabolism disorders. The rs9997745 mutant of ACSL1 increases the risk of metabolic syndrome, related to type 2 diabetes^[45,46]. In addition, ACSL1 expression is increased in mononuclear cells in type 1 diabetic patients^[47],

suggesting that ACSL1 may be involved in the pathogenesis of diabetes.

ACSL AND NERVOUS SYSTEM DISEASES

Abnormal lipid metabolism can also cause neurological disease. ACSL6 predominates in nerve cells and ACSL6 insufficiency can lead to neuronal degeneration^[48]. Studies have shown that ACSL6 over-expression leads to neurite outgrowth in rats, while silencing ACSL6 inhibits axon outgrowth of mouse neural cells^[28,48]. ACSL6-induced activation of acetylcholinesterase may be involved in this process, as acetylcholinesterase promotes neural differentiation^[49]. ACSL4 has been reported to be correlated with X-linked mental retardation, which leads to a higher incidence of mental disorders^[50]. Recent studies have shown that, like ACSL6, ACSL4 showed specificity for polyunsaturated fatty acids, such as AA and eicosapentaenoic acid, which may contribute to changes in function of specific tissue^[4,28].

ACSL AND OTHER DISEASES

Studies have shown that ACSLs also play a role in other diseases. Methylation of ACSL3 5'-CpG island (CGI) is thought to be correlated with maternal exposure to polycyclic aromatic hydrocarbons in the air, which is also closely correlated with the increased incidence of asthma, suggesting that ACSL3 may

Table 1 Long-chain acyl-CoA synthetase expression and pathways involved in liver diseases

Liver diseases		mRNA expression	Pathways	Ref.
ACSL1	HF	↓	PPAR/NF-κB/p65	Xin <i>et al</i> ^[39] and Pyper <i>et al</i> ^[42]
	NAFLD	↓	TGF	Uto <i>et al</i> ^[43]
ACSL3	NAFLD	↓	LXR/RXR	Dong <i>et al</i> ^[44]
ACSL4	HCV	↓	--	Yao <i>et al</i> ^[17]
	HCC	↑	P38/cAMP	Liang <i>et al</i> ^[32]
ACSL5	NAFLD	↑	--	Stepanova <i>et al</i> ^[41]
	NAFLD	↑	Caspase	Reinartz <i>et al</i> ^[40]

ACSL: Long-chain acyl-CoA synthetase; HF: Hepatic fibrosis; NAFLD: Non-alcoholic fatty liver disease; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PPAR: Peroxisome proliferator-activated receptor; NF-κB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells; TGF: Transforming growth factor; LXR: Liver X receptor; RXR: Retinoid X receptor; cAMP: 3'-5'-cyclic adenosine monophosphate.

play a role in asthma^[51,52]. Studies have shown that ACSL4 stimulates the release of prostaglandin E₂ by arterial smooth muscle cells, which promotes atherosclerosis formation, suggesting the involvement of ACSL4 in atherosclerosis^[23,53,54]. ACSL4 has also been linked to uterus abnormalities, Alport syndrome and elliptocytosis^[55,56]. ACSL5 transcription is elevated in systemic lupus erythematosus (SLE), and the treatment of SLE with corticosteroids decreased ACSL5 transcription^[36]. Patients with inflammatory bowel disease showed over-expression of ACSL1 and ACSL5 mRNAs in the terminal ileum and colon^[57].

CONCLUSION

Long-chain Acyl-CoA synthetase plays a crucial role in fatty acid metabolism. Fatty acids, saturated or unsaturated, are a major source of energy in humans and are essential, particularly unsaturated fatty acids. Fatty acids need to be activated to form acyl-CoA before they enter a metabolic pathway, including both anabolic and catabolic pathways. Recent research on ACSLs mainly focused on the effect on fatty acid metabolism and less on the influence on cell proliferation and apoptosis. The mechanisms involved in fatty acid metabolism and cell proliferation and apoptosis are unclear. Further studies to elucidate the function of ACSL enzymes would be highly beneficial.

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Basic Study

Degradation of intestinal mRNA: A matter of treatment

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conditions: different species (human and rat), varying temperature (storage on crushed ice or room temperature), time point of dissection of the submucous-mucous layer from the smooth muscle (before or after storage), different rinsing methods (rinsing with Medium, PBS, RNALater or without rinsing at all) and different regions of the gut (proximal and distal small intestine, caecum, colon and rectum). The total RNA from different parts of the gut (rat: proximal and distal small intestine, caecum, colon and rectum, human: colon and rectum) and individual gut layers (muscle and submucosal/mucosal) was extracted. The quality of the RNA was assessed by micro capillary electrophoresis. The RNA quality was expressed by the RNA integrity number which is calculated from the relative height and area of the 18 S and 28 S RNA peaks. From rat distal small intestine qPCR was performed for neuronal and glial markers.

RESULTS: RNA obtained from smooth muscle tissue is much longer stable than those from submucosal/mucosal tissue. At RT muscle RNA degrades after one day, on ice it is stable at least three days. Cleaning and separation of gut layers before storage and use of RNALater, maintains the stability of muscle RNA at RT for much longer periods. Different parts of the gut show varying degradation periods. RNA obtained from the submucosal/mucosal layer always showed a much worse amplification rate than RNA from muscle tissue. In general RNA harvested from rat tissue, either smooth muscle layer or submucosal/mucosal layer is much longer stable than RNA from human gut tissue, and RNA obtained from smooth muscle tissue shows an increased stability compared to RNA from submucosal/mucosal tissue. At RT muscle RNA degrades after one day, while the stability on ice lasts at least three days. Cleaning and separation of gut layers before storage and use of RNALater, maintains the stability of muscle RNA at RT for much longer periods. Different parts of the gut show varying degradation periods. The RNA from muscle and submucosal/mucosal tissue of the proximal small intestine degrades much faster than the RNA of distal small intestine, caecum or colon

Abstract

AIM: To characterize the influence of location, species and treatment upon RNA degradation in tissue samples from the gastrointestinal tract.

METHODS: The intestinal samples were stored in different medium for different times under varying

with rectum. RNA obtained from the submucosal/mucosal layer always showed a much more reduced amplification rate than RNA from muscle tissue [β -Tubulin III for muscle quantification cycle (C_p): 22.07 ± 0.25 , for β -Tubulin III submucosal/mucosal C_p : 27.42 ± 0.19].

CONCLUSION: Degradation of intestinal mRNA depends on preparation and storage conditions of the tissue. Cooling, rinsing and separating of intestinal tissue reduce the degradation of mRNA.

Key words: Intestinal RNA; Degradation; Storing conditions; Flushing; Cooling

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Core tip: The quality of RNA is crucial for an appropriate RNA analysis. Especially when working with human material, precious samples will often be used for different purposes and can therefore not be frozen immediately. Gut tissue is especially fragile and RNA degrades rapidly if not treated adequately. Under these aspects RNA degradation of different gut sections and gut wall layers regarding their treatment was investigated in this study. Storage, rinsing and preparation conditions are essential for RNA stability. Sufficient and permanent cooling as well as the removal of bacterial contamination leads to a reduced degradation in muscle tissue of the gut.

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INTRODUCTION

RNA isolation followed by PCR analysis has become a major tool in modern research^[1-4]. Working with RNA requires the control of RNA integrity^[5]. This is a critical first step in obtaining meaningful gene expression data. Working with low quality RNA might have a significant impact on the experimental results of downstream applications, which are often labour-intensive, time-consuming, and expensive. Using intact RNA is a key factor for the successful application of modern molecular biological methods, such as qRT-PCR or microarray analysis^[6,7]. Samples are usually immediately frozen to avoid prolonged storage under suboptimal conditions. The analysis and evaluation of human samples depends on a stable supply and often only small amounts of tissue can be provided. In many cases these rare samples are used for various

experiments^[8], including the isolation of living cells^[9], thus making it necessary to use alternative storage and transportation conditions of vital tissue. Especially in cases, where the samples are received at different locations, or even from abroad, transportation time might exceed 24 h. In these cases, the samples cannot simply be frozen; they have to be transported as vital tissue on crushed ice, which is not an ideal condition for RNA conservation. The integrity of the sample RNA depends strongly on the individual tissue^[10]. When working with the sensible gut tissue, several major drawbacks came into the focus of attention. The amount of digestive enzymes and a broad range of microorganisms^[11,12] are rendering the gut a problematic tissue. Moreover, it contains different compartments or layers (muscle, submucosal and mucosal layer)^[13] with individual properties. In the actual study the integrity of the RNA was investigated concerning transportation or storage time and condition as well as the influence of the gut content upon the individual layer.

MATERIALS AND METHODS

Human postnatal gut

Intestinal tissue from children (age ranged from two month to two years) was obtained from two surgical facilities. A total of ten gut tissue samples of the colon which were sectioned in one centimetre segments were included in the study. These sections were analysed as a whole or separated into muscle and submucosal/mucosal layer. All samples were collected after written informed consent of the parents of the patients, according to the Declaration of Helsinki and with the approval of the local ethics committee.

Animals

Sprague Dawley rats of one to two weeks of age were used. Rats were killed by decapitation and the small intestine, caecum and colon were removed. The small intestine was sectioned into equal segments. Animal protocols were approved by the internal Veterinary Inspection Office in Mannheim.

Experimental approaches

For all experiments performed in this study human or rat tissue samples (each one cm long) were used. The individual experiments differ concerning species, location, gut layer, storage conditions (time and temperature), time of separation of the layers and different rinsing methods.

In the first experiment the differences in RNA degradation between human and rat gut tissues have been investigated. The tissues were temporarily stored in a Hepes buffered minimal essential medium (MEM-Hepes) with a stable glutamine dipeptide (Glutamax) on ice. After different storage times the tissue was

separated in muscle and submucosal/mucosal layer: 6 h, 24 h and 72 h. The 0 h samples were frozen immediately in liquid nitrogen and stored until further processing for RNA extraction, as described below.

The second experiment was performed to demonstrate the influence of the temperature. The samples from rat small intestine were flushed first in PBS, then separated into muscle and submucosal/mucosal layer and then stored for different times (0 h, 0.5 h, 1 h, 1.5 h, 3 h, 6 h, 8 h, 24 h, 48 h and 72 h) in MEM-Hepes either on ice or at room temperature (RT) before storage until further processing for RNA extraction, as described below.

A third experiment was designed to investigate more precisely the influence of separation of the gut different layers prior to processing upon RNA degradation. The samples from rat small intestine were rinsed in MEM-Hepes with antibiotics (Gentamycin and Metronidazol) before being temporarily stored (0 h, 0.5 h, 1 h, 1.5 h, 3 h, 6 h, 8 h, 24 h, 48 h and 72 h) in MEM-Hepes (Glutamax) on ice or at RT. After storage samples were separated in muscle and submucosa/mucosa. A second group of tissue was investigated where muscle and submucosal/mucosal layer had been separated before storage (same times as above) until further processing for RNA extraction, as described below.

A forth experiment should evaluate the influence of different rinsing solutions. The samples from rat small intestine were rinsed after dissection in PBS or MEM-Hepes with antibiotics (Gentamycin and Metronidazol), in RNALater or not rinsed at all. Then they were temporarily stored in the individually used rinsing solutions on ice or at RT. The tissue was separated in muscle and submucosal/mucosal layer at different times: 0 h, 8 h, 24 h and 48 h before storage until further processing for RNA extraction, as described below.

A fifth experiment was performed to analyze whether the gut region alters the effect of RNA degradation. The whole rat gut was separated into proximal and distal small intestine, caecum, colon and rectum. Then the gut tissues were temporarily stored in a Hepes buffered MEM with a stable glutamine dipeptide (Glutamax) at RT. The tissue was separated into muscle and submucosal/mucosal layer at different times (0 h, 8 h, 24 h and 48 h) before stored until further processing for RNA extraction, as described below.

RNA extraction

Total cellular RNA was extracted from one centimetre tissue segments using an "Isolate RNA Mini Kit" (Bioline, Luckenwalde, Germany) following the manufacturer's protocol. The contaminating genomic DNA was digested with DNase I (Invitrogen, Karlsruhe, Germany). RNA concentration was measured spectrophotometrically using an infinite M200 micro plate reader (Tecan, Mainz-Kastel, Germany).

Degradation testing

RNA quality assessment was tested by micro capillary electrophoresis using an Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) with the RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) following the manufacturer's protocol^[14]. The RNA quality was determined by the RNA integrity number (RIN), which is calculated from the relative height and area of the 18 S and 28 S RNA peaks and follows a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the best preserved^[15,16]. For the degradation test we used RNA with a concentration of 50 ng/ μ L.

Real time PCR analysis of gene expression

BioScript TM was used to generate cDNAs (Bioline) from 100 ng RNA according to the manufacturer's protocol. For real time PCR the SensiMixSYBR Low Rox Kit (Bioline) was used on a MX3005 (Stratagene). 100 ng cDNA was used for each sample. The individual tests were performed in triplicates and repeated 3 times. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The PCR conditions were as follows: initial denaturation 10 min, 95 °C; 40 cycles of denaturation; 30 s, 95 °C; annealing, 30 s, 55 °C; elongation 30 s, 72 °C. The primer sequences (F: forward; R: reverse) were as follows: r-S100 (F: 5'-TTGCCCTCATTGATGTCTTCCA-3', R: 5'-TCTGCCTTGATTCTTACAGGTGAC-3') from Lisachev *et al.*^[17], r-Tubulin- β III (F: 5'-AGACCTACTGCATCGACAATGAAG-3', R: 5'-GCTCATGGTAGCAGACACAAGG-3') from Schwindt *et al.*^[18], PGP9.5 (F: 5'-CCCTGAAGACAGAGCCAAAGTG-3', R: 5'-GAGTCATGGGCTGCCTGAA-3'), and GAPDH (F: 5'-GTATGACTTACCCACGGCAAGT-3', R: 5'-TTCCCGTTGATGCCAGCTT-3') from Du *et al.*^[19].

Statistical analysis

For time course measurement on degradation effect of muscle and submucosal/mucosal human and rat tissue in student's *t*-test was applied, after testing for normal distribution. The results were considered significant with a $P < 0.05$.

The response RINs were fit using standard least-square regression using JMP version 10 to explore the impact of temperature, tissue, time, species and tissue separation of the gut tissue in muscle and submucosal/mucosal layer before or after incubation. The contribution of the different formulation variables was compared using analysis of variance (ANOVA) at $P < 0.05$ significance level.

RESULTS

Degradation of muscle and submucosal/mucosal layer RNA of human and rat gut at different points in time

Degradation of RNA was assessed using an Agilent 2100 bioanalyzer. This approach was applied to a large collection of electrophoretic RNA measurements. The

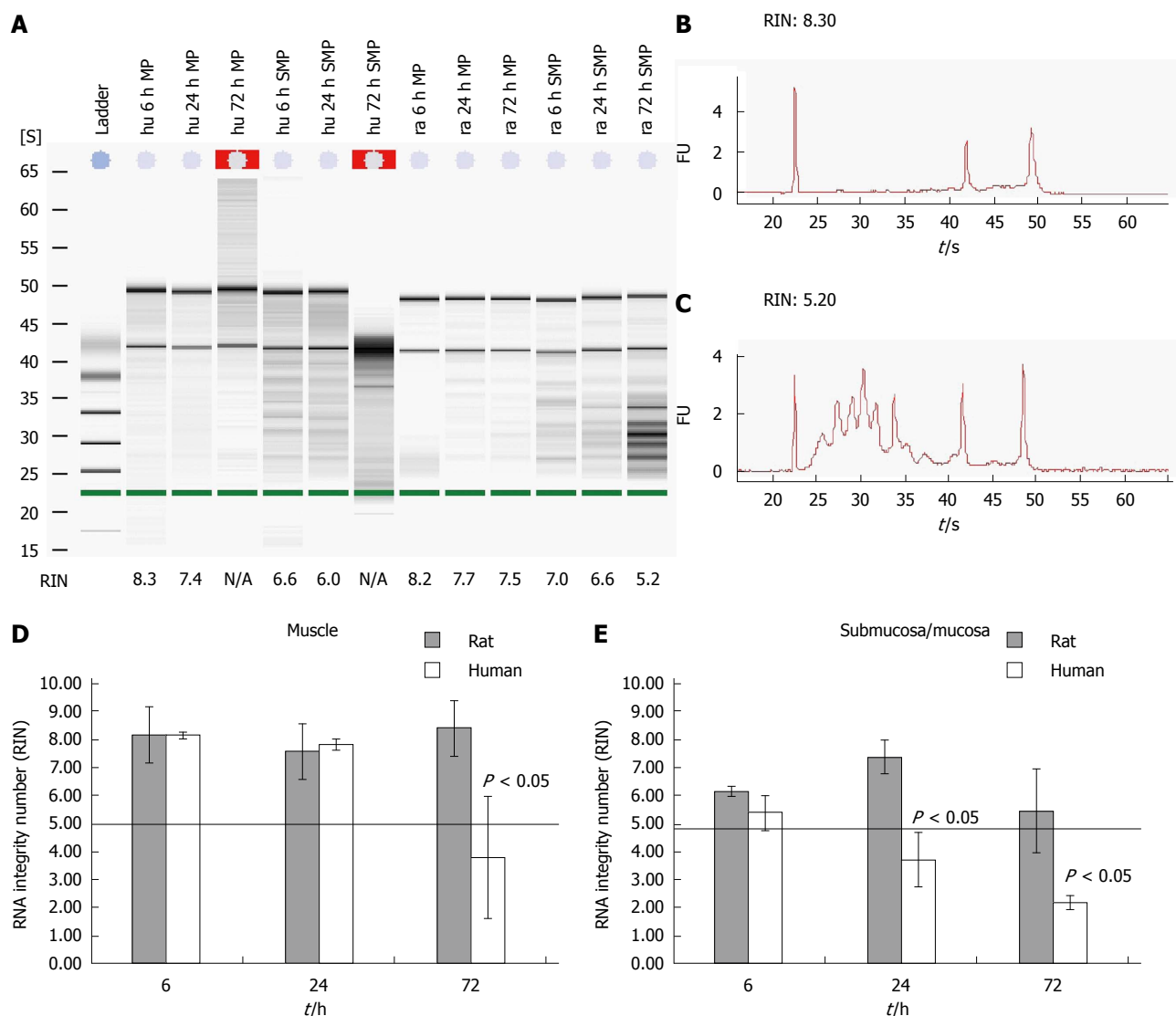


Figure 1 Time course of degradation effect on muscle and submucosal/mucosal human and rat tissue. Human and rat gut samples were stored in MEM-Hepes on ice and separated in smooth muscle and submucosal/mucosal layer (muscle, mucosa) at different points in time. RNA from the tissues was isolated and degradation (RIN) of the different samples was examined. A: The figure shows a typical gel for human and rodent samples with different integrities. First the ladder, followed by 3 human muscle samples (hu MP), another 3 samples from human mucous layer (hu SMP) all from different points in time. The last 6 samples were comparable samples from rat (ra); B and C: Typical electrophoretic RNA measurements obtained from an Agilent 2100 bioanalyzer displaying different RIN values for intact (8 and 3 respectively; B) and partly degraded RNA (5 and 2 respectively; C); RIN values from both rat and human tissues from different compartment and time points are depicted in (D) and (E). Data are presented as the mean \pm SE. $n = 10$.

resulting algorithm is a user-independent, automated and reliable procedure for standardization of RNA quality control that allows the calculation of an RNA integrity number (RIN)^[16]. A minimum level of RNA integrity is necessary to obtain reliable qPCR data. RNA with a RIN below five is highly degraded and should not be used within further qPCR experiments^[10]. The needs for microarray experiments are even higher. Here you need a RIN higher than 7. Moreover, a RIN that might be adequate for a 3' amplification might not work for a 5' amplification. To test the RNA degradation progress of different tissues gained from human and rodent gut on ice, the samples were separated into muscle and submucosal/mucosal layers (Figure 1A-C). The RNA of the human muscle was intact for more than 24 h. After 6 h the RNA had a RIN of 8.2 ± 0.11

and a RIN of 7.8 ± 0.2 after 24 h. After 72 h the RNA of the muscle was degraded and the RIN dropped to 3.8 ± 2.2 (Figure 1D). The RNA of the submucosal/mucosal layer degraded much faster than the RNA of the muscle layer. The RIN of the submucosal/mucosal layer showed a still acceptable value of 5.4 ± 0.6 after six h and was fully degraded after 24 h with a RIN of 3.7 ± 1.0 and a RIN of 2.2 ± 0.2 after 72 h (Figure 1E).

Comparing rat and human tissue reveals significant differences. While the RIN of rat smooth muscle tissue still reaches 8.4 ± 0.4 (Figure 1D) after 72 h of storage, the human tissue yields not more than a RIN of 3.8 ± 2.2 (Figure 1D). Rat derived submucosal/mucosal layer RNA degraded even faster, already after 24 h. At this point of time the RNA delivered a RIN value of 7.4 ± 0.6 . After 48 and 72 h the RIN was still

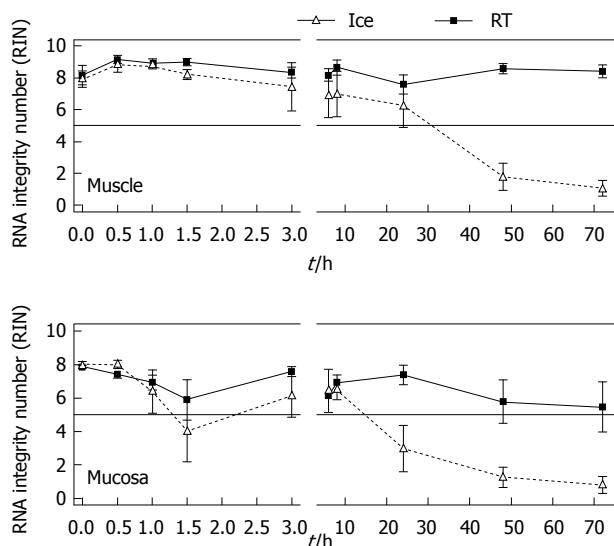


Figure 2 Time and temperature based measurement of RNA integrity of isolated RNA from rat muscle and submucosal/mucosal tissue of the gut. Pieces of rat gut were stored in MEM-Hepes on ice or at room temperature (RT) and at different points in time samples were separated into smooth muscle and submucosal/mucosal layer (muscle, mucosa). The RNA integrity number was assessed for the individual samples. Data are presented as the mean \pm SE. $^{\circ}P < 0.05$ and $n = 6$.

acceptable with values around 5.5 ± 2 (Figure 1E). Human derived submucosal/mucosal tissue degraded already after 6 h with a RIN of 5.4 ± 0.6 and 3.7 ± 1 after 24 h. At this time point the RNA of human submucosal/mucosal layer is completely degraded compared to RNA of rat submucosal/mucosal layer (Figure 1E).

Influence of temperature and time upon RNA degradation in different gut layers

In order to investigate when exactly the RNA of the gut degrades and which role temperature and time are playing, a more detailed analysis was performed with rat derived intestinal tissue. The amount of tissue was not limited. Furthermore individual short periods of time could be explored. Rat tissue samples were collected and stored temporarily in MEM-Hepes on ice or at RT until RNA was extracted and measured. Full thickness samples as well as individual muscle or submucosal/mucosal layers were analysed after separation (Figure 2). Muscle RNA kept its stability for more than 72 h when stored on ice. Here the RIN values varied between 9.2 at “zero” time and 7.6 after 72 h. The RNA quality was still sufficient for further experiments. After 24 h at RT the RNA was 6.2 ± 1.3 , after 48 h it dropped to 1.8 ± 0.9 and after 72 h the RIN reached a value of 1.1 ± 0.5 (Figure 2). The RNA of the submucosal/mucosal layer remained intact on ice over 72 h with a RIN of 5.5 ± 1.5 while at room temperature it already degraded after 8 h. Here the RIN dropped to 6.5 ± 0.6 and 3 ± 1.4 after 24 h. Degradation kept on until it reached a value of 1.0 ± 0.5 at 72 h (1.3 ± 0.6 , 48 h, Figure 2).

Influence of separation and rinsing prior to the extraction process

To study whether a special tissue treatment will protect the RNA from degradation, the gut was rinsed with sterile PBS before separating the layers into muscle and submucosal/mucosal tissue. The tissues were either separated immediately before or after storage. Storage took place in MEM-Hepes, either on ice or at RT. After different times the RNA of the samples was isolated and the degradation evaluated. After 72 h the RNA of the muscle layer showed similar RIN values on ice (RIN 7.3 ± 1.2) as at RT (RIN 8.6 ± 0.4) (Figure 3A). So it seems that rinsing and separating the tissue in muscle and submucosal/mucosal layers, prevents the RNA of the muscle in case the cold chain is interrupted. Submucosal/mucosal RNA degraded after 24 h on ice. The RNA after 24 h had a RIN of 6.2 ± 1.1 and of 4.3 ± 1.6 after 48 h, respectively 3.2 ± 1.6 after 72 h (Figure 3A). At RT the RNA of the submucosal/mucosal layer degraded at 8-h incubation in medium and yielded a RIN of 4.9 ± 1.3 , while there was a complete degradation to be seen after 24 h with a RIN of 1 ± 0.2 (Figure 3A). Separating muscle and submucosal/mucosal layer makes no difference to the quality of the RNA from the submucosal/mucosal layer in contrast to the muscle.

A multifactorial analysis of variances of the RIN in response to several factors: temperature, tissue, time, species and tissue separation of gut in muscle and submucosal/mucosal layer before or after incubation was undertaken. To identify the impact factors to the RIN, an analysis of variance (ANOVA) at $P = 0.05$ significant level was used.

The factors temperature, tissue, time, species and tissue separation of the gut tissue in muscle and submucosal/mucosal layer before or after incubation had all a significant influence on the RIN of the RNA (Figure 3B). The factors submucosal/mucosal layer, RT, time, human and separation of gut tissue after incubation time, influence the RIN negatively (Figure 3B). The most important factor for the RIN with the t ratio of 12.52 is the tissue, followed by the time with a t ratio of 10.66, temperature with a t ratio of 6.93, species with a t ratio of 6.23 and finally the separation of the tissue in muscle and submucosal/mucosal layer before or after incubation with a t ratio of 3.63.

Use of different media for rinsing and storage of the tissue

As the previous experiments revealed that rinsing the specimen protects the muscle from RNA degradation, the rinsing media might also be critical. We therefore tried various media for the initial rinsing step. Three different rinsing solutions (MEM+, PBS or RNALater) were used and the individual tissues were compared with tissues which were not rinsed at all and just stored in MEM plus. The tissues were stored in the same media in which they have been rinsed for various

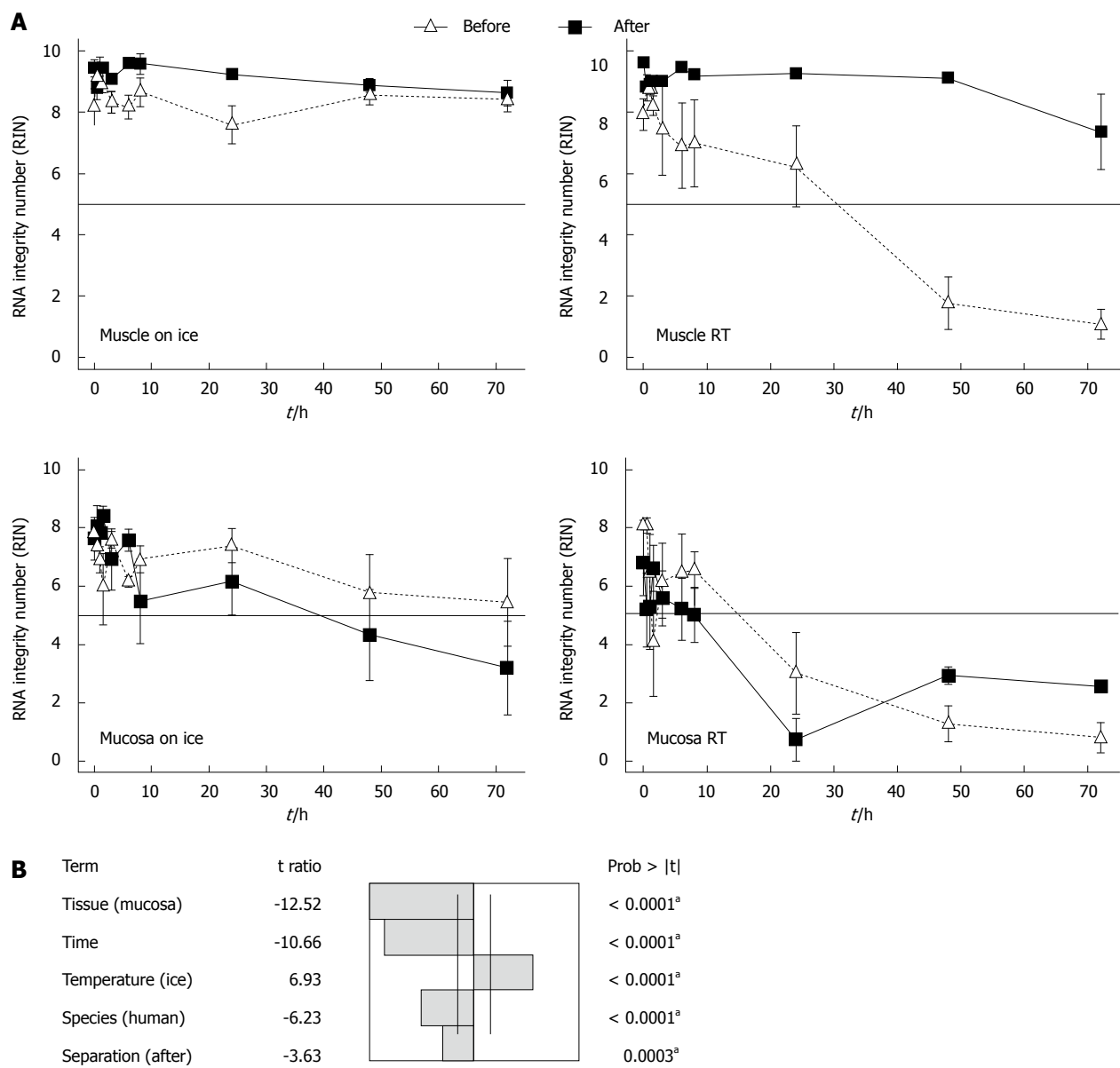


Figure 3 Time course of RNA integrity from isolated RNA from muscle and submucosal/mucosal tissue with an initial separation or with separation after storage. A: In the experiment “before” the gut from rat was first rinsed with PBS and separated into muscle and submucosal/mucosal layer and then the samples were stored in medium on ice or at room temperature (RT). In the experiment “after” the tissue of the gut was stored in medium on ice or at RT and at different points in time samples were washed with PBS and separated in muscle and submucosal/mucosal layer. The RNA integrity number was measured. Data are presented as the mean \pm SE; ^a $P < 0.05$ and $n = 6$; B: A fitting of the factors: temperature, tissue, time, species and tissue separation of the gut in muscle and submucosal/mucosal layer before or after incubation was calculated. To identify the impact factors upon the RIN, an analysis of variance (ANOVA) at ^a $P < 0.05$ significant level was used and $n = 6$. RIN: RNA integrity number.

incubation times (0 h, 8 h, 24 h and 48 h) on ice or at RT. Regarding the tissues stored in control medium, MEM+ or PBS no visible difference after incubation were observed (Figure 4). However, the tissue that had been washed and stored in RNeasy did shrink and became very sticky. This tissue was therefore very hard to dissect in muscle and submucosal/mucosal layer. Due to this heavy sticking the tissue could not be pipetted appropriately. Moreover the tissue was very fragile and easily destroyed. In this experiment it could clearly be demonstrated that the RNA from muscle and submucosal/mucosal layer was best protected

by cooling (Figure 4). As soon as the samples were stored on ice, only one significant difference in the muscle was seen after 24 h. Here the tissue stored in RNeasy had a significant ($P = 0.001$) higher RIN. All other washing and storing methods displayed no significant different effects, even after 48 h. However, when the samples were stored at RT, we noticed significant differences for the muscle up to 24 h (Figure 4). The samples which were washed and stored in RNeasy had a much better RIN value (eight) than the control samples. Also the samples stored in MEM+ or PBS had a RIN below five. This effect was even

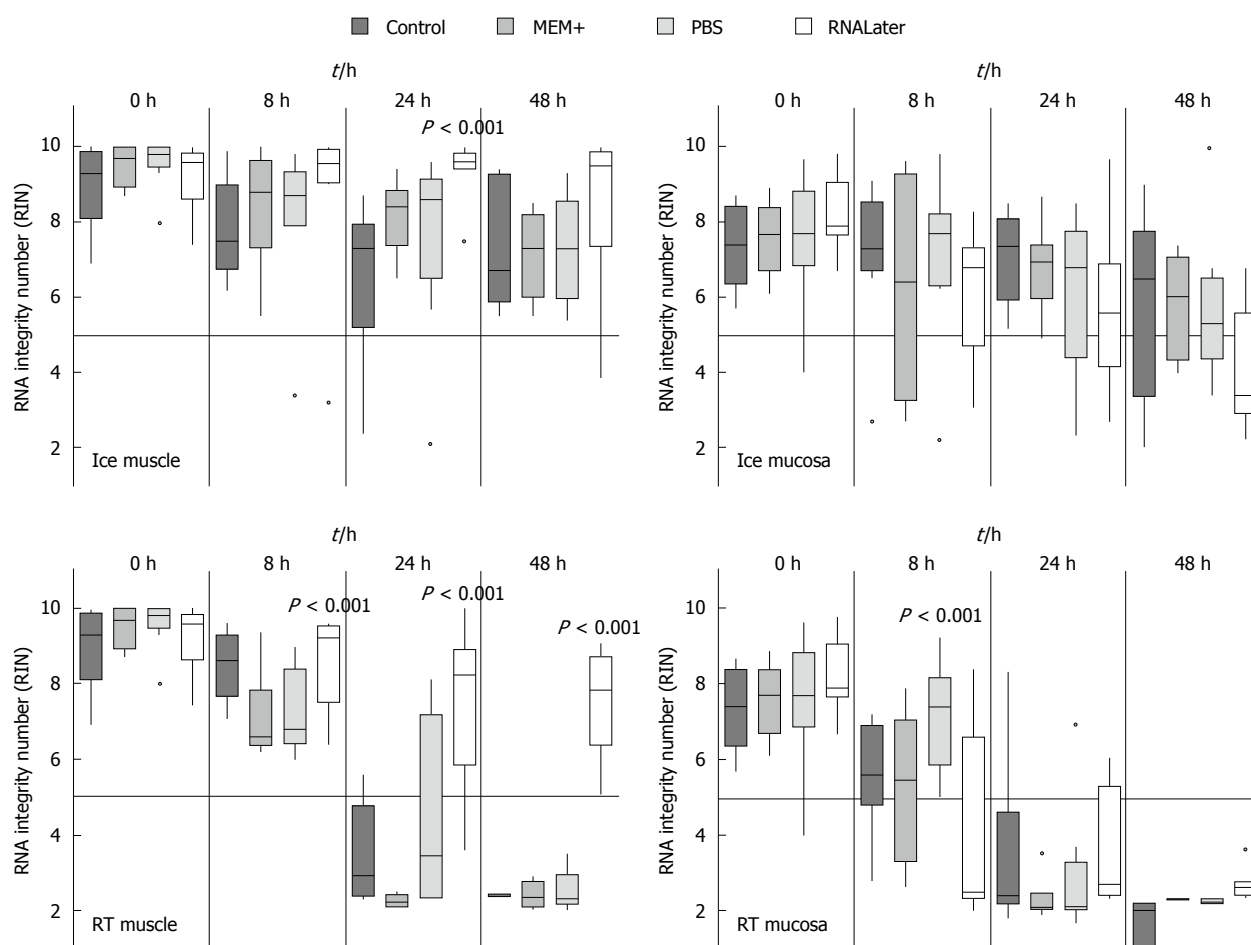


Figure 4 Time course of RNA integrity from isolated RNA from muscle and submucosal/mucosal tissue treated with different methods. Rat gut was rinsed with different media and stored in medium on ice or at room temperature at different points in time (0 h, 8 h, 24 h and 48 h). Then the samples were separated in smooth muscle (muscle) and submucosal/mucosal layer (mucosa). The RNA Integrity number was measured and data are presented as the mean \pm SE and tested for statistical significance using one way ANOVA. The results were considered significant with $P < 0.001$ and $n = 9$. RIN: RNA integrity number.

more pronounced after 48 h, where the RNALater samples still had a RIN value greater seven, while all others provided only completely degraded RNA (Figure 4).

In the submucosal/mucosal layer samples a similar protective effect of cooling could be demonstrated as seen in the muscle tissue, while there was no difference between the individual media used. At RT RNALater did not have any protective effect on submucosal/mucosal tissue. Interestingly, the best protection effect after 8 h could be seen with PBS ($P = 0.001$). After 24 h and 48 h, the RNA was completely degraded in submucosa/mucosal tissue in all rinsing solutions (Figure 4).

RNA integrity in different parts of the gut

To verify subsequently whether the individual sections of the intestine have an influence on the stability of RNA and whether bacteria or digestive enzymes, which are different in each section, have influence upon the RNA degradation, they were examined separately on RNA degradation. To test this, the intestine was separated into proximal and distal small intestine, caecum and colon with the rectum. These pieces were

stored at RT about 0 h, 8 h, 24 h and 48 h in MEM and separated into muscle and submucosal/mucosal layer afterwards. It could be clearly demonstrated that the RNA from the proximal small intestine degraded much faster ($P = 0.001$, 8 h), as those from other locations (Figure 5). This RNA had already a RIN value about 5 after 8 h in both submucosal/mucosal and muscle layer while the other sections still had a value above seven or ten in both muscle and submucosal/mucosal layer respectively. The muscle RNA after 24 h was degraded only in the proximal small intestine. All other parts had a RIN higher than 5. After 48 h there was no difference between either region or tissue type, all RNA was degraded (Figure 5).

Expression of neuronal and glial genes in submucosal/mucosal and muscle layer

In order to analyse whether the expression of genes from the enteric nervous system (ENS) depends on RNA degradation, the RNA of muscle and of submucosal/mucosal tissue with different RIN's (two to nine) was analysed with qPCR. Neuronal and glial genes (β -Tubulin III, PGP9.5 and S100), as well as

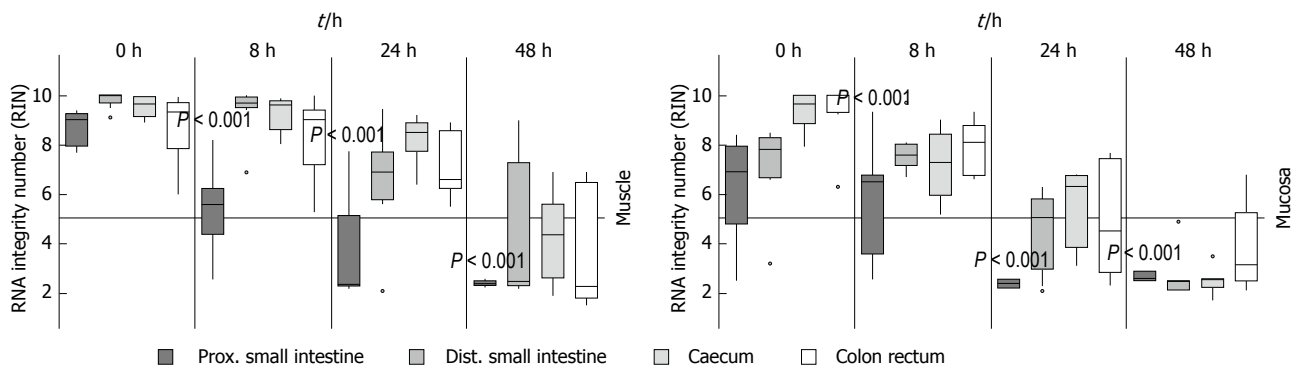


Figure 5 Measurement of RNA Integrity in different parts of the gut. Rat gut was separated in different parts (proximal small intestine, distal small intestine, caecum and colon with rectum) and then the samples were stored in medium at room temperature and at different points in time (0 h, 8 h, 24 h and 48 h) the tissue was separated in smooth muscle (muscle) and submucosal/mucosal layer (mucosa). The RNA Integrity numbers were calculated and data presented as the mean \pm SE and tested for statistical significance using ANOVA. The results were considered significant with $P < 0.001$ and $n = 9$.

Table 1 Real time PCR from muscle and submucosal/mucosal layer of individual gut layers

Tissue	Gene	Cp (RIN = 9)	Cp (RIN = 2)
Muscle	β -Tubulin III	22.07 \pm 0.25	24.41 \pm 0.22
Submucosal/mucosal	β -Tubulin III	27.42 \pm 0.19	26.00 \pm 0.93
Muscle	PGP 9.5	20.28 \pm 0.09	23.67 \pm 0.58
Submucosal/mucosal	PGP 9.5	25.43 \pm 0.54	25.28 \pm 0.58
Muscle	S100b	26.65 \pm 0.47	29.07 \pm 0.02
Submucosal/mucosal	S100b	28.53 \pm 0.63	29.75 \pm 1.30
Muscle	GAPDH	18.85 \pm 0.05	18.68 \pm 0.08
Submucosal/mucosal	GAPDH	18.93 \pm 0.04	19.53 \pm 0.20

The expression patterns in terms of cycles do depend on the RNA integrity number (RIN). Cp (RIN = 9): Quantification cycle by qPCR from RNA with a RIN of 9; Cp (RIN = 2): Quantification cycle by qPCR from RNA with a RIN of 2.

GAPDH as an independent gene for degradation^[6] were investigated. When the RIN of the muscle RNA decreases, the quantification cycle (Cp) of the qPCR increases for the neuronal and glial genes (Table 1). So it seems that the Cp from muscle layer depends on the RIN.

The Cp of the submucosal/mucosal layer with a RIN of nine equals the RIN of five from muscle tissue. The Cp from the submucosal/mucosal layer seems not to be dependent on the RIN of the RNA.

DISCUSSION

While quality control of RNA is routinely being performed prior to microarray-based gene expression profiling, it is often missing with regard to PCR-based quantification methods. Indeed, even on degraded RNA samples, a nice amplification curve can be obtained^[20]. Nevertheless, excellent RNA quality is essential to obtain reliable results. The inclusion of samples with degraded RNA may influence the statistical analysis and hence the interpretation of gene expression levels in relation to biological and/or clinical data. Results should reflect real biological differences and not differences due to poor RNA integrity^[21].

The gut is a highly delicate tissue that is filled with digestive enzymes and a microbiome of varying quality and quantity. This leads to a rapid post mortem or even post dissection degradation of the whole tissue. Obviously, human RNA degrades faster than the one derived from rat. RNA from post mortem brain is much more stable than RNA from post mortem intestinal tissue^[22]. We demonstrated similar differences between rat and human tissue from surgical gut samples. The storage and dissection before RNA processing changes their individual quality. This was most pronounced in the smooth muscle from both rat and human.

Generally, RNA from the muscle layer remains much longer stable than RNA from the submucosal/mucosal layer. In the submucosal/mucosal layer usually more immune cells are to be found, and due to the neighbourhood to the lumen and the loose arrangement of the tissue the bacterial translocation takes place earlier and to a greater extent.

We have shown in the intestinal samples that measures such as rinsing the tissue and separating the bowel in its submucosal/mucosal and muscle compartments, reduces especially the RNA degradation in the muscle. If the cold chain is not interrupted the RNA of the smooth muscle remains stable for a minimum of 72 h. In contrast, rinsing and separating the tissue wall does not protect the RNA from submucosal/mucosal layers. Here, only cooling reduces degradation. These effects might even be more pronounced when the intestinal tissue is inflamed^[3]. We could also demonstrate that different rinsing methods have no impact upon RNA integrity, if the tissue is cooled. But the rinsing method has an influence if the tissue is stored at RT. RNALater prevents RNA degradation in the muscle at all-time points investigated, while also PBS had a preventing influence upon the submucosal/mucosal layer after 8 h at RT. This might be due to an inhibition of degrading enzymes which needs calcium or magnesium for being active^[23,24].

Not only that RNA from the intestine degrades faster than RNA from blood cells, or cell lines, there

are also local differences. The RNA of the proximal part of the small intestine (duodenum) degrades much faster than the ones from the distal parts of the small intestine (ileum and jejunum) or the large bowel (caecum and colon with rectum). This demonstrates that digestive enzymes might also play an important role for RNA degradation. Other groups showed that the RNA from ileum and colon degraded slower than the one from jejunum^[10]. This is consistent with our data concerning the duodenum. In rodents, proximal small intestine samples might still harbour parts of the pancreas, which is (unless in human) - not arranged in a compact organ. Bits of pancreatic tissue are embedded in the mesenterial wall along the duodenum, and might easily be overseen during dissection. Pancreatic tissue will yield a surplus of degrading enzymes. Moreover, degradation of the RNA does also depend on the amount of connective tissue or fat, or as in our case, in increased enzyme activity^[10]. It can lead to a faster degradation of the RNA^[22] or caused by inadequate sample processing and storage^[20]. Using RNase inhibitors can reduce the problem of RNA degradation, but is not consistent among individuals and RNA degradation even occurred when frozen samples were thawed immediately before nucleic acid extraction^[25].

Adequate sample storage in medium as well as cooling reduces RNA degradation at least in muscle and submucosal/mucosal layer.

Also it is known that in some mammalian tissue or blood or even the food in the gut contains inhibiting factors which interferes with the PCR assays^[26-30]. So there might be some unknown factors in the submucosal/mucosal layer that can produce a false negative result for the qPCR. In general, rinsing, cooling and separation make the difference concerning RNA quality. While RNeasy increases RNA quality, its use makes only sense provided a sole RNA extraction is performed. As soon as the tissue has to be processed further, *i.e.*, by separating the individual gut wall layers, RNeasy interferes negatively with the dissection process. In order to avoid misinterpretations, especially with a limited amount of crucial material, optimal pre-treatment and processing is an indispensable prerequisite to obtain reliable and standardized RNA analysis data.

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COMMENTS

Background

The RNA quality of each sample has a great influence on a variety of experimental measurement methods. It is therefore important to test the quality before the individual experiment starts, especially in tissues from the gastrointestinal tract, where both secreted enzymes or fecal and microbial

content of the gut can interfere with the quality of RNA.

Research frontiers

RNA from the gastrointestinal tract is particularly interesting in various diseases, be it inflammatory or developmental. A standardized protocol for optimal RNA treatment allows the comparison of results from different laboratories in a more reliable way.

Innovations and breakthroughs

A detailed protocol of sample preparation for optimal RNA harvest is provided. Different species have been compared and variations and influences of both species and gut segments investigated.

Applications

The provided data can be used for a more standardized investigation of RNA changes in health and disease, based on optimized techniques.

Terminology

RNA degradation means that the information sequence of the RNA is destroyed and can therefore not be used for a reliable identification of specific genes, involved in diseases or developmental changes.

Peer-review

This is a study in which the authors analysed the effect of different treatment and storage conditions of intestinal tissue upon the RNA quality derived from these tissues. The results are interesting and show that cooling and separation of tissue prevents the RNA of degradation.

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Basic Study

Effect of entacapone on colon motility and ion transport in a rat model of Parkinson's disease

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Author contributions: Li LS and Zhu JX designed the study; Li LS and Liu CZ carried out most of the experiments and analyzed the data; Xu JD, Zheng LF, Feng XY and Zhang Y performed the *I_{sc}* experiments; Li LS and Zhu JX wrote the paper.

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Abstract

AIM: To study the effects of entacapone, a catechol-O-methyltransferase inhibitor, on colon motility and electrolyte transport in Parkinson's disease (PD) rats.

METHODS: Distribution and expression of catechol-O-methyltransferase (COMT) were measured by immunohistochemistry and Western blotting methods. The colonic smooth muscle motility was examined *in vitro*

by means of a muscle motility recording device. The mucosal electrolyte transport of PD rats was examined by using a short-circuit current (*I_{sc}*) technique and scanning ion-selective electrode technique (SIET). Intracellular detection of cAMP and cGMP was accomplished by radioimmunoassay testing.

RESULTS: COMT was expressed in the colons of both normal and PD rats, mainly on the apical membranes of villi and crypts in the colon. Compared to normal controls, PD rats expressed less COMT. The COMT inhibitor entacapone inhibited contraction of the PD rat longitudinal muscle in a dose-dependent manner. The β_2 adrenoceptor antagonist ICI-118,551 blocked this inhibitory effect by approximately 67% ($P < 0.01$). Entacapone increased mucosal *I_{sc}* in the colon of rats with PD. This induction was significantly inhibited by apical application of Cl^- channel blocker diphenylamine-2, 2'-dicarboxylic acid, basolateral application of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter antagonist bumetanide, elimination of Cl^- from the extracellular fluid, as well as pretreatment using adenylate cyclase inhibitor MDL12330A. As an inhibitor of prostaglandin synthetase, indomethacin can inhibit entacapone-induced *I_{sc}* by 45% ($P < 0.01$). When SIET was applied to measure Cl^- flux changes, this provided similar results. Entacapone significantly increased intracellular cAMP content in the colonic mucosa, which was greatly inhibited by indomethacin.

CONCLUSION: COMT expression exists in rat colons. The β_2 adrenoceptor is involved in the entacapone-induced inhibition of colon motility. Entacapone induces cAMP-dependent Cl^- secretion in the PD rat.

Key words: Parkinson disease; Entacapone; Colon motility; Ion transport; Catechol-O-methyltransferase

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Core tip: Entacapone, a catechol-O-methyltransferase (COMT) inhibitor, is an emerging drug for Parkinson's disease (PD) patients. However, patients experience gastro-intestinal side effects with entacapone treatment and the reason for this is unknown. This study for the first time proved that COMT is expressed in normal and PD rat colons and that entacapone can inhibit PD rat muscle contraction through the β_2 adrenoceptor. It was also discovered that entacapone can induce cAMP-dependent Cl^- secretion in PD rats and that endogenous prostaglandin is involved in this process. These findings provided histological evidence of COMT in the colon, establishing an experimental basis for the mechanism of entacapone-induced PD gastro-intestinal side effects.

Li LS, Liu CZ, Xu JD, Zheng LF, Feng XY, Zhang Y, Zhu JX. Effect of entacapone on colon motility and ion transport in a rat model of Parkinson's disease. *World J Gastroenterol* 2015; 21(12): 3509-3518 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3509.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3509>

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurologic disorders, affecting approximately 1% of individuals older than 60 years^[1]. L-DOPA is currently the most effective treatment for PD^[2,3]. However, most patients with late-stage disease, or those who have been treated with L-DOPA for over 3 to 5 years, will develop "chronic syndromes", such as desensitization to treatment or dyskinesia^[4-6]. Entacapone, a catechol-O-methyltransferase (COMT) inhibitor, is an emerging drug that overcomes these problems^[7-9]. However, patients experience gastro-intestinal side effects with entacapone treatment, especially diarrhea, with some patients displaying constipation and abdominal pain. Approximately 1% of patients discontinue entacapone treatment due to abdominal pain. Discontinuing use of entacapone for several days alleviates the adverse gastro-intestinal effects, although the mechanism for this observation is not clear. Abdominal pain can reach a moderate level, and taking large doses (> 1600 mg/d) of entacapone increases the occurrence and severity of the abdominal pain; the reason for this phenomenon is also unknown^[10-12].

We hypothesized that the adverse gastro-intestinal effects of entacapone on PD patients was due to changes in intestinal smooth muscle motility, or to effects on ion transport in the intestinal epithelium. COMT is expressed in several tissues in humans and rodents. However, the precise localization of COMT in normal and PD rat intestine is not clear; there are also no studies reporting how entacapone affects ion transport in the intestines of PD patients or whether it affects smooth muscle motility^[13-16]. Due to the above factors, this study utilized *in vitro* rat colon smooth

muscle and mucosal samples to examine the precise localization of COMT in the rat colon. The effect of entacapone on colon smooth muscle and epithelial ion transport in PD rats was investigated. In addition, the main reasons responsible for entacapone-induced adverse intestinal effects were explored. This study provides experimental evidence for the prevention and treatment of these side effects.

MATERIALS AND METHODS

Experimental animals

The animals were purchased from the Department of Animal Science of Capital Medical University. Specific-pathogen-free (SPF) male SD rats with body weights of 200-300 g were randomly grouped. The animals were kept at room temperature, with normal light/dark cycle exposure and 24-h water and food access until the day of the experiment. The experiment was approved by the Laboratory Animal Welfare Committee.

Main reagents and preparation

The main reagent, entacapone, is a product of the Orion Corporation. Indomethacin, TTX, bumetanide, and DPC were purchased from Sigma (St. Louis, MO). All chemical reagents were dissolved in dimethyl sulfoxide (DMSO), and the DMSO volume fraction did not exceed 0.1%. Preliminary experiments showed that the solvent did not alter basic electrophysiologic parameters. In addition, NaCl, KCl, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KH_2PO_4 , NaHCO_3 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and glucose were purchased from Sigma.

Preparation of the main reagents

The Krebs-Henseleit solution (K-HS) was prepared as follows: sodium chloride 117 mmol/L, potassium chloride 4.7 mmol/L, calcium chloride 2.5 mmol/L, magnesium chloride 1.2 mmol/L, sodium bicarbonate 24.8 mmol/L, monopotassium phosphate 1.2 mmol/L, and glucose 11.1 mmol/L. For Cl^- -free K-HS, sodium gluconate, potassium gluconate, calcium gluconate, and magnesium gluconate were used to replace the sodium chloride, potassium chloride, calcium chloride, and magnesium chloride, respectively.

Establishing the PD rat model

Male SD rats weighing 210 to 240 g were selected. First, the weights were taken, and 0.4 mL of 10% chloral hydrate/100 g body weight was injected for anesthetization. The animals were placed on a Kopf stereotaxic apparatus. According to the coordinates, the positions posterior to the frontal suture 5.6 mm, shifted 2 mm laterally, or AP = -5.6 (posterior to the frontal suture is negative and anterior to the frontal suture is positive), ML = ± 2 mm (right is negative and left is positive) were located by adjusting the guide bars and were marked with a marker. Four microliters of 6-OHDA (2 $\mu\text{g}/\mu\text{L}$), a total of 8 μg of drug, was administered at an even speed of 1 $\mu\text{L}/\text{min}$. The

Table 1 Selected primary antibodies in the study

Antigen	Immunizing antigen	Host species	Dilution		Source/Catalog No.
			IHC	WB	
COMT	Synthetic peptide corresponding to amino acid residues 60-76 of rat COMT conjugated to KLH	Rabbit	1:200	1:1000	Santa Cruz/sc-25844
COMT	Synthesized peptide derived from human COMT	Rabbit	1:400	1:1000	Sigma/SAB4500401
GAPDH	A synthetic peptide containing 314-333 amino acids of mouse GAPDH with a 407972 gene ID	Rabbit	-	1:5000	Sigma/G9545
NF	Pellet of pig brain cold stable proteins after depolymerization of microtubules	Mouse	1:400	-	Abcam/ab7794
NF	Recombinant C-terminal segment of rat NF-M	Rabbit	1:400	-	Novus/NB300-133

COMT: Catechol-O-methyltransferase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NF: Neurofilament.

Table 2 Selected secondary antibodies in the study

Antibody	Conjugation	Dilution	Source/Catalog No.
Goat anti-rabbit IgG	Alexa Fluor 488	1:100	Beyotime/A0423
Goat anti-rabbit IgG	Alexa Fluor 488	1:100	Beyotime/A0428
Donkey anti-rabbit IgG	IRDye™800	1:10000	Rockland/16747

needle was kept at the position for 2 min. The needle was then lifted slowly, and a small amount of saline was scattered to hydrate the incision. A dried saline saturated gelatin sponge was used to seal the incision. Penicillin powder was scattered before the skin was closed by suture. Then, an intraperitoneal injection of penicillin (0.5 mL/animal) was administered. The procedures of the normal control group were the same as those of the experimental model group, except that the normal control group was administered saline.

Immunohistochemistry and protein blotting methods

Western blot analysis and immunohistochemistry were performed as previously described^[17,18]. Information on antibodies used in this study is summarized in Tables 1 and 2.

Recording of rat colon smooth muscle contraction

The rats were killed and then distal colon was quickly removed. The luminal contents of the colon were washed with Krebs solution. The 1st to 4th segments of the distal colon were collected. Each segment was cut open along the mesentery border and spread evenly over silica gel with the apical membrane facing down. The samples were surrounded by ice-cold Krebs solution to maintain tissue activity. The entire colon was cut into 2-mm-wide, 1-1.5-cm-long strips along the longitudinal or circular muscles. Surgical sutures were used to tie a piece of thread to each end of the muscle strip and connect one end to a specimen support rod and the other end to a pressure transducer. The muscle strips were incubated in 10 mL of Krebs solution at a constant temperature (37 ± 0.5 °C) under continuous gas ventilation (95% oxygen and 5% carbon dioxide). After applying 1g of resting muscle tension, the change in length was recorded by

the pressure transducer. With 2000 mg of weight for calibration, intensity of muscle strip contraction was measured with mg as the basic unit. After the muscle strips were adapted in a water bath for 120 min, the autonomous contraction intensity was officially recorded.

Tissue preparations for measuring the *I_{sc}*

For the preparation of the rat colon mucosa sample, roughly 7 cm of colon from above the rectal lymph node was obtained (this lymph node is often located roughly 3 cm up the rectum). Using fine tip forceps under a dissection microscope to carefully separate the submucosa, muscularis, and serosa from the mucosa with blunt dissection, a thin layer of tissue that included epithelia and some residual connective tissue was obtained. This tissue comprised the rat colon mucosa sample.

I_{sc} recording

This experiment utilized a constant temperature perfusion system to record the *I_{sc}* *in vitro*. The colon mucosa sample was placed in the Ussing chamber of the perfusion system. Both sides of the colon mucosa were injected with 5 mL of K-HS, and 95% oxygen and 5% carbon dioxide were administered simultaneously to maintain the solution pH at approximately 7.4. The samples were incubated in a 37 °C water bath for 30 min to allow the electrical parameters to stabilize. A voltage clamp was utilized to maintain the epithelial voltage at zero potential, short-circuiting the tissue, and the transepithelial current measured at this time was the *I_{sc}*^[19].

Measurement of extracellular Cl⁻ flux

This experiment utilized SIET (BIO-001A, YoungerUSA Sci. & Tech. Corp., United States). The noninvasive microelectrode (model XY-H-01) used for measuring the Cl⁻ concentration and flux was provided by Xuyue (Beijing) Sci. & Tech. Co., Ltd. The tip of the Cl⁻ selective microelectrode was filled with a 15-25 μm chloride selective liquid ion exchanger column (LIX) and was subsequently filled with an approximately 10 mmol/L electrolyte solution column. The Cl⁻ selective

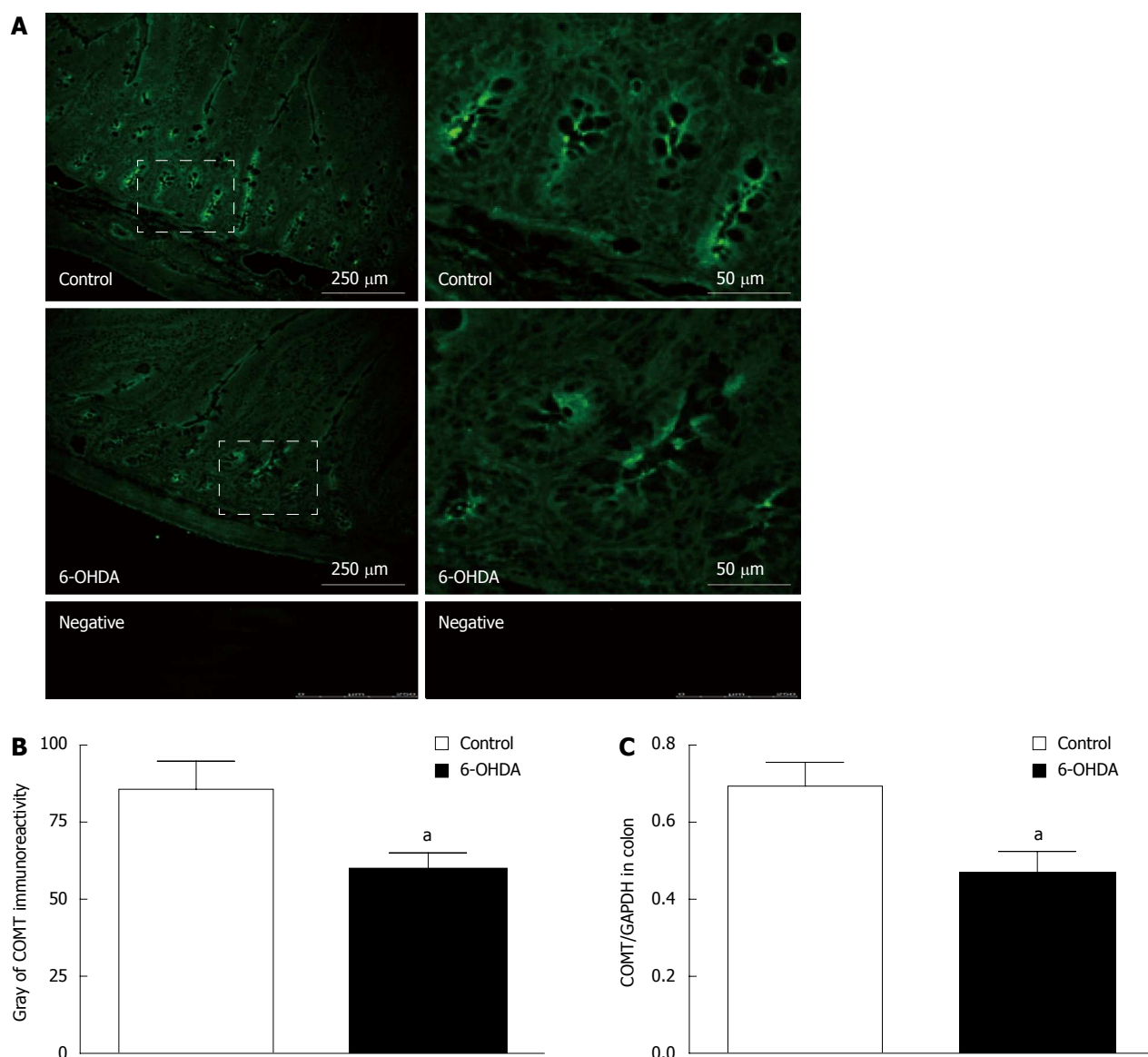


Figure 1 Characteristics of catechol-O-methyltransferase localization in normal and 6-OHDA Parkinson's disease model rats. A: The COMT immunoreactivity in colon; B: The gray changes of COMT immunoreactivity in colon; C: The changes of expression of COMT in colonic mucosal preparations in 6-OHDA PD rats ($n = 8$, ^a $P < 0.05$ vs control). COMT: Catechol-O-methyltransferase; PD: Parkinson's disease.

microelectrode must be calibrated before use; only Nernstian Slope > 56 mV/decade electrodes can be used, and the distance between the Cl^- electrode and cells must be controlled to approximately $30 \mu\text{m}$. The absolute concentration, flux direction, and flux rate difference before and after drug treatment were compared^[20].

cAMP/cGMP detection

The rat colon mucosa samples were collected and placed in a 37°C K-HS solution gassed with 5% CO_2 and 95% O_2 for incubation; each sample weighed approximately 150 mg. After incubation and stabilization, the tissue was treated with 0.9% NaCl and indomethacin ($10 \mu\text{mol/L}$) for 5 min. Next, 200 $\mu\text{mol/L}$ entacapone was added to react for 15 min. To observe the effect of various drugs on the entacapone-

induced intracellular cAMP/cGMP level changes, after drug pretreatment, the tissues were cut quickly, with excess water blotted on filter paper, and flash frozen in liquid nitrogen. The tissues were then homogenized on ice and centrifuged ($10620 \times g$, 5 min) for analysis. Intracellular cAMP/cGMP detection was accomplished using a commercial radioimmunoassay kit (RIA) (Beijing Huaying Biotechnology Co. Ltd., Beijing, China).

Statistical analysis

GraphPad Prism 5.0 software was used for the statistical analysis. Data are expressed as the mean \pm SE. The means between two groups were analyzed by *t*-test. When the variance was unequal, the Wilcoxon rank-sum test was used for comparison of the means of two groups. The significance level was set at $P = 0.05$.

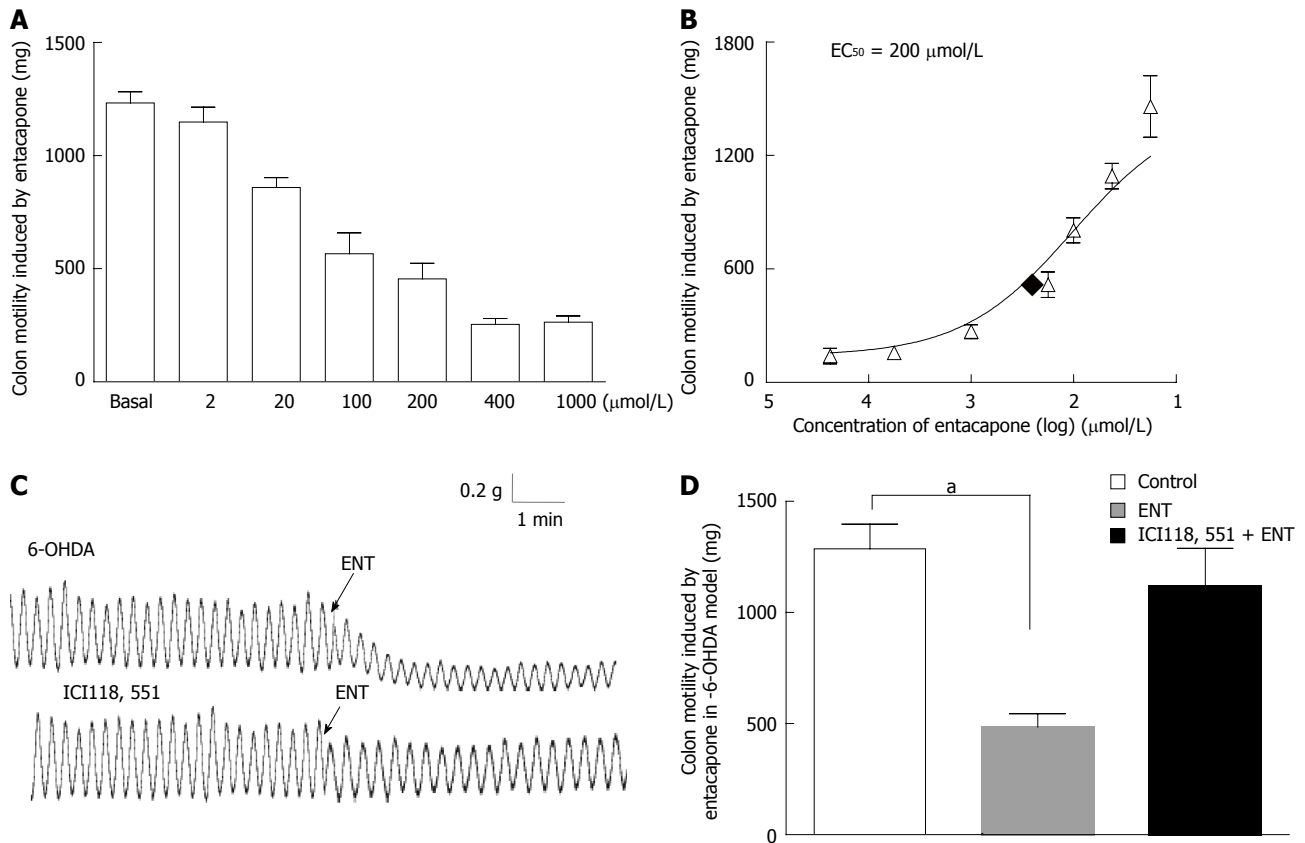


Figure 2 Effect of entacapone on colon smooth muscle motility in 6-OHDA Parkinson's disease rats. A: Inhibitory effect of entacapone on colon longitudinal muscle contraction; B: Dose-response curve effect of entacapone on colon smooth muscle motility in 6-OHDA rats ($n = 30$); C: Representative tracing of strips when using entacapone (ENT) and β_2 receptor antagonist ICI-118,551 (1.0×10^{-5} mol/L) in 6-OHDA rats; D: The role of β_2 receptor in entacapone-induced effect of smooth muscle inhibition ($n = 9$, $^aP < 0.05$ vs control).

RESULTS

Characteristics of COMT localization in normal and 6-OHDA PD model rats

To provide morphological evidence, this study used immunofluorescence labeling and immunohistochemistry, utilizing rat colon cryosections to determine the localization of COMT. It was discovered that in both normal and 6-OHDA PD model rats, COMT was expressed more abundantly on the villi and crypts at the apical membrane (Figure 1A). The gray analysis of COMT immunoreactivity in normal rats (78.23 ± 4.63 , 48 fields of vision COMT from 8 rats) was higher than that in the PD group (60.27 ± 3.96 , 48 fields of vision COMT from 8 rats) (Figure 1B).

Immunoblotting similarly confirmed that COMT is localized in colon tissue. Compared to normal rats, 6-OHDA PD model rats expressed slightly less COMT. A quantitative comparison using an internal reference by GAPDH showed a 27% decrease in COMT expression, from 0.67 ± 0.04 to 0.49 ± 0.06 in PD model rats (Figure 1C) ($n = 8$, $P < 0.05$).

Effect of entacapone on colon smooth muscle motility in 6-OHDA PD rats

Entacapone concentrations of 2 μmol/L, 20 μmol/L, 100 μmol/L, 200 μmol/L, 400 μmol/L and 1000 μmol/L

were used individually to observe their effects on intestine longitudinal muscle in PD model rats. It was observed that longitudinal muscle contraction was decreased from baseline (1233 ± 50.13 mg) to 1150 ± 64.96 mg, 859.9 ± 43.54 mg, 566.8 ± 92.6 mg, 450.6 ± 74.86 mg, 304.9 ± 53.7 mg and 307.98 ± 63.4 mg, respectively ($n = 30$, Figure 2A). This result indicates that entacapone has an inhibitory effect on colon longitudinal muscle contraction with a dose-dependent pattern, by EC_{50} of 200 μmol/L (Figure 2B). However, the different doses of entacapone did not significantly alter the PD rat colon circular muscle (data not shown).

To investigate the mechanism of entacapone-induced smooth muscle inhibition, the samples were pretreated with β_2 adrenoceptor antagonist ICI-118,551 (10^{-5} mol/L), and it was found that entacapone-induced colon longitudinal muscle inhibition was decreased by 67%, from 782.4 ± 34.24 mg to 258.39 ± 39.47 mg ($n = 9$, Figure 2C and D). However, with pretreatment with α adrenoceptor antagonist phentolamine (10^{-5} mol/L), entacapone-induced smooth muscle inhibition was not affected ($n = 9$). We also found that using acetylcholine (10^{-6} mol/L) for pretreatment or administering entacapone before adding acetylcholine did not significantly affect entacapone-induced smooth muscle inhibition (data

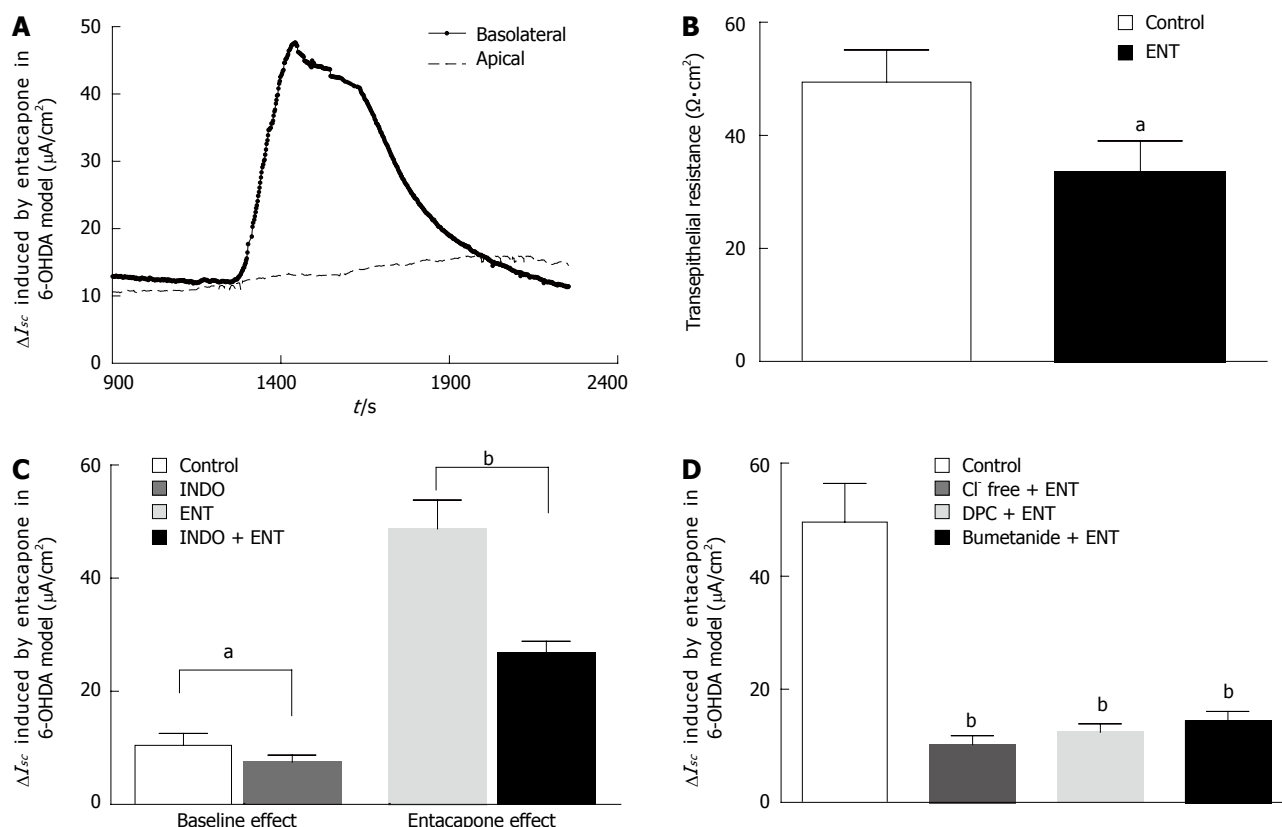


Figure 3 Entacapone-induced I_{sc} response in colonic mucosa specimens in 6-OHDA Parkinson's disease rats. A: The short circuit current (I_{sc}) changes induced by apical and basolateral using of ENT (200 $\mu\text{mol/L}$); B: Basolateral application of ENT (200 $\mu\text{mol/L}$) induced transepithelial electric resistance ($n = 13$); C: Effect of indomethacin (INDO) (10 $\mu\text{mol/L}$) on the entacapone (ENT)-caused I_{sc} changes with 6-OHDA PD rats ($n = 9$); D: Summary of the effects of pretreatment by removing Cl^- from the extracellular fluid, the apical use of DPC (1 mmol/L) (a type of Cl^- channel inhibitor), and the basolateral use of $\text{Na}^+/\text{K}^+-2\text{Cl}^-$ co-transporter (NKCC) antagonist bumetanide (100 $\mu\text{mol/L}$) on the ENT-caused I_{sc} changes in mucosal preparations of colon in 6-OHDA PD rats ($n = 9$, $^aP < 0.05$, $^bP < 0.01$ vs control).

not shown). The results show that entacapone might exert its inhibitory effects on PD rat colon smooth muscle through the β_2 adrenoceptor.

Effect of entacapone on colon mucosa I_{sc} change in 6-OHDA PD rats

PD rat colon mucosa samples were placed in the I_{sc} apparatus. After approximately 30 min, the basal electrical activity of the samples reached stability. In this experiment, all mucosa samples were pretreated with TTX to eliminate the effects from residual neural activity. The basal potential difference, I_{sc} , and transepithelial resistance (Rt) were -0.8 ± 0.3 mV, 10.1 ± 3.2 $\mu\text{A/cm}^2$, and 49.6 ± 5.4 $\Omega \cdot \text{cm}^2$, respectively. Adding 200 $\mu\text{mol/L}$ entacapone to the basolateral (serosal) side can induce a 47.69 ± 8.91 $\mu\text{A/cm}^2$ increase in I_{sc} (Figure 3A). Simultaneously, a significant decrease in the Rt from 49.6 ± 5.4 $\Omega \cdot \text{cm}^2$ to 36.84 ± 3.94 $\Omega \cdot \text{cm}^2$ ($n = 13$, $P < 0.05$) was observed (Figure 3B). When entacapone was added to the apical (mucosal) side, there was no significant change in the I_{sc} (Figure 3A).

To investigate the effect of endogenous prostaglandin on entacapone-induced I_{sc} changes in PD rat colons, the cyclooxygenase (COX) inhibitor indomethacin (10 $\mu\text{mol/L}$) was added to the basolateral side of the

mucosa for observation. As shown in Figure 3C, after pretreatment with indomethacin, the mucosal basal I_{sc} decreased from 10.1 ± 2.14 $\mu\text{A/cm}^2$ to 7.28 ± 2.06 $\mu\text{A/cm}^2$ ($n = 9$, $P < 0.05$), reaching a 28% reduction; the pretreatment was able to decrease the entacapone-induced I_{sc} from 47.69 ± 8.91 $\mu\text{A/cm}^2$ to 26.14 ± 2.67 $\mu\text{A/cm}^2$ ($n = 9$, $P < 0.01$), reaching a 45% reduction. These results show that endogenous prostaglandin was involved in PD rat colon basal I_{sc} formation and entacapone-induced I_{sc} change.

Generally speaking, an upward I_{sc} curve reflects the occurrence of cation absorption or anion secretion with electrogenic features. In order to study the properties of the electric activities and involvement in ion channels or transporters, the following experiment was performed (Figure 3D). Epithelial Na^+ channel inhibitor amiloride (10 $\mu\text{mol/L}$) or DIDS (Ca^{2+} -dependent Cl^- channel inhibitor, 200 $\mu\text{mol/L}$) was applied to the apical side of mucosa, and it was observed that they did not reduce the entacapone-induced increase of the I_{sc} ($n = 9$, data not shown). However, removal of Cl^- from the mucosa sample bath buffer could reduce entacapone-induced I_{sc} by 79%, from 47.69 ± 8.91 $\mu\text{A/cm}^2$ to 10.01 ± 2.53 $\mu\text{A/cm}^2$ ($n = 9$, $P < 0.01$). When DPC (1 mmol/L) (a nonselective Cl^- channel blocker) was added to the apical side or when bumetanide (100 $\mu\text{mol/L}$) (a $\text{Na}^+/\text{K}^+-2\text{Cl}^-$ cotransporter inhibitor) was

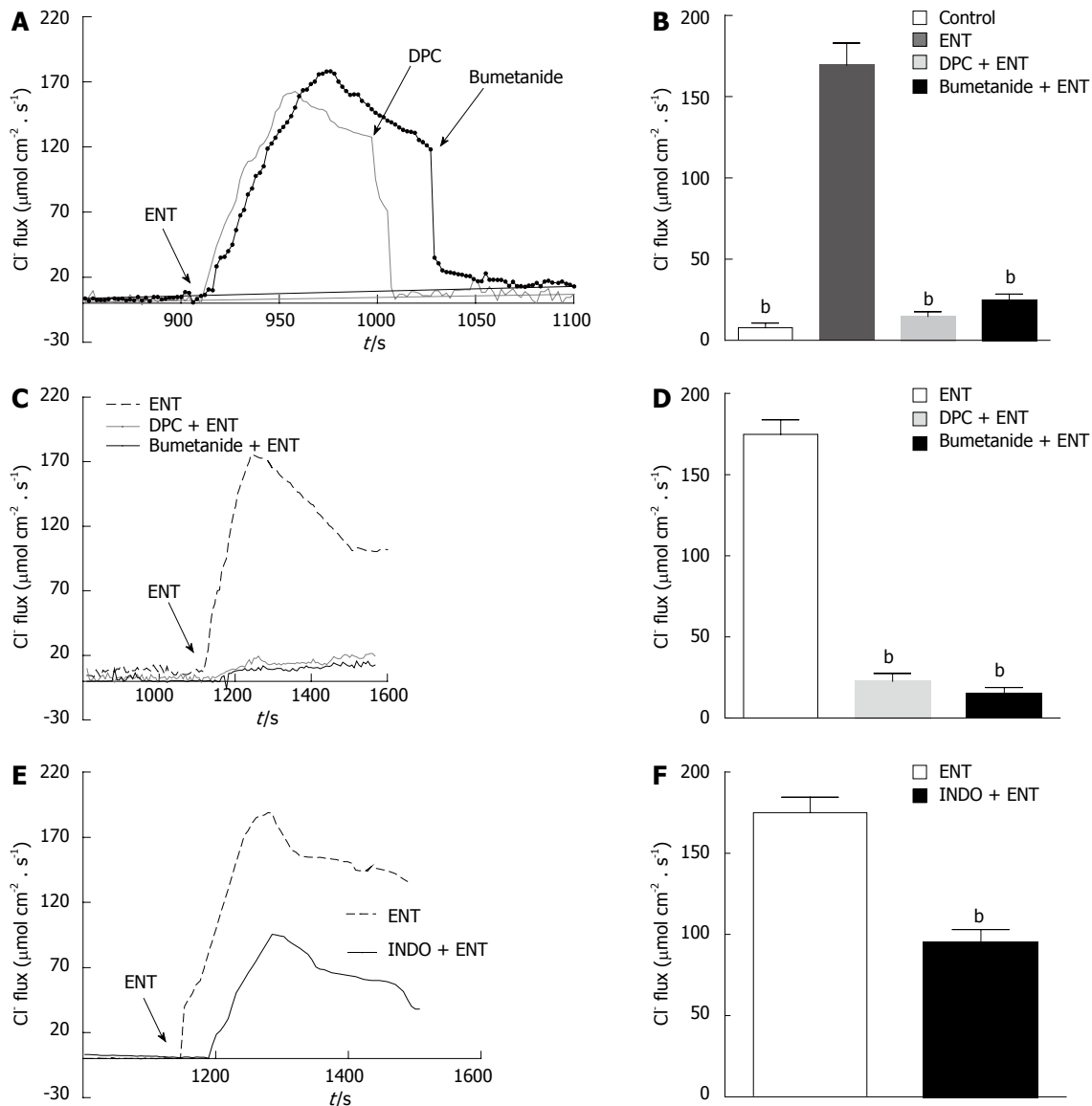


Figure 4 Utilizing scanning ion-selective electrode technique to observe the effect of entacapone on colon mucosa Cl^- -flux in 6-OHDA rats. A: Application of entacapone (ENT) (200 $\mu\text{mol/L}$), diphenylamine-2, 2'-dicarboxylic acid (DPC) (1 mmol/L) and bumetanide (100 $\mu\text{mol/L}$), causing the typical performance of Cl^- flux in colon specimens in 6-OHDA Parkinson's disease (PD) rats; B: Generalization of the roles of bumetanide and DPC on the Cl^- -flux caused by ENT in colon specimens of 6-OHDA PD rats ($n = 10$); C: Application of ENT (200 $\mu\text{mol/L}$), preprocessing by bumetanide or DPC, causing the typical performance of Cl^- flux in colon specimens of 6-OHDA PD rats; D: Generalization of Cl^- -flux caused by ENT after treatment by bumetanide and DPC in colonic specimens ($n = 9$); E: Application of ENT (200 $\mu\text{mol/L}$), and preprocessing by indomethacin (INDO) (10 $\mu\text{mol/L}$), causing the typical performance of Cl^- flux in colon specimens in 6-OHDA PD rats; F: Generalization of the roles of INDO on the Cl^- -flux caused by ENT ($n = 9$, $^bP < 0.01$ vs control).

added to the basolateral side, this also significantly reduced the entacapone-induced I_{sc} by 74% and 68% to 12.4 ± 3.78 ($n = 9$, $P < 0.01$) and 15.26 ± 3.52 $\mu\text{A}/\text{cm}^2$ ($n = 9$, $P < 0.01$), respectively. These results show that entacapone-induced I_{sc} changes in PD rat colons are mainly mediated by Cl^- secretion.

Utilizing SIET to observe the effect of entacapone on colon mucosa Cl^- -flux in 6-OHDA rats

SIET is a new research method. Using specialized ion-selective electrodes, it is possible to obtain information regarding ion movements. Thus, we used this method to directly measure entacapone-induced PD rat colon Cl^- transport.

As Figure 4 shows, using Cl^- sensitive electrodes

at the basolateral side of PD rat colon mucosa, a small, stable Cl^- -flux can be recorded. The addition of entacapone (200 $\mu\text{mol/L}$) could induce a significant Cl^- -flux from the basolateral side of the colon mucosa into the cells, showing a significant increase from 6.37 ± 3.01 $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ to 172.3 ± 14.86 $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$. This Cl^- -flux could be inhibited by DPC (1 mmol/L) and bumetanide (100 $\mu\text{mol/L}$), reducing the concentrations to 13.69 ± 3.12 $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ and 22.3 ± 3.72 $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$, respectively (Figure 4A and B). If pretreated with DPC and bumetanide, similar results were observed; the entacapone-induced flux was reduced to 22.07 ± 3.59 $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ and 18.35 ± 3.1 $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ ($n = 10$, $P < 0.001$), separately (Figure 4C and D). The pretreatment by indomethacin (10 $\mu\text{mol/L}$) was able

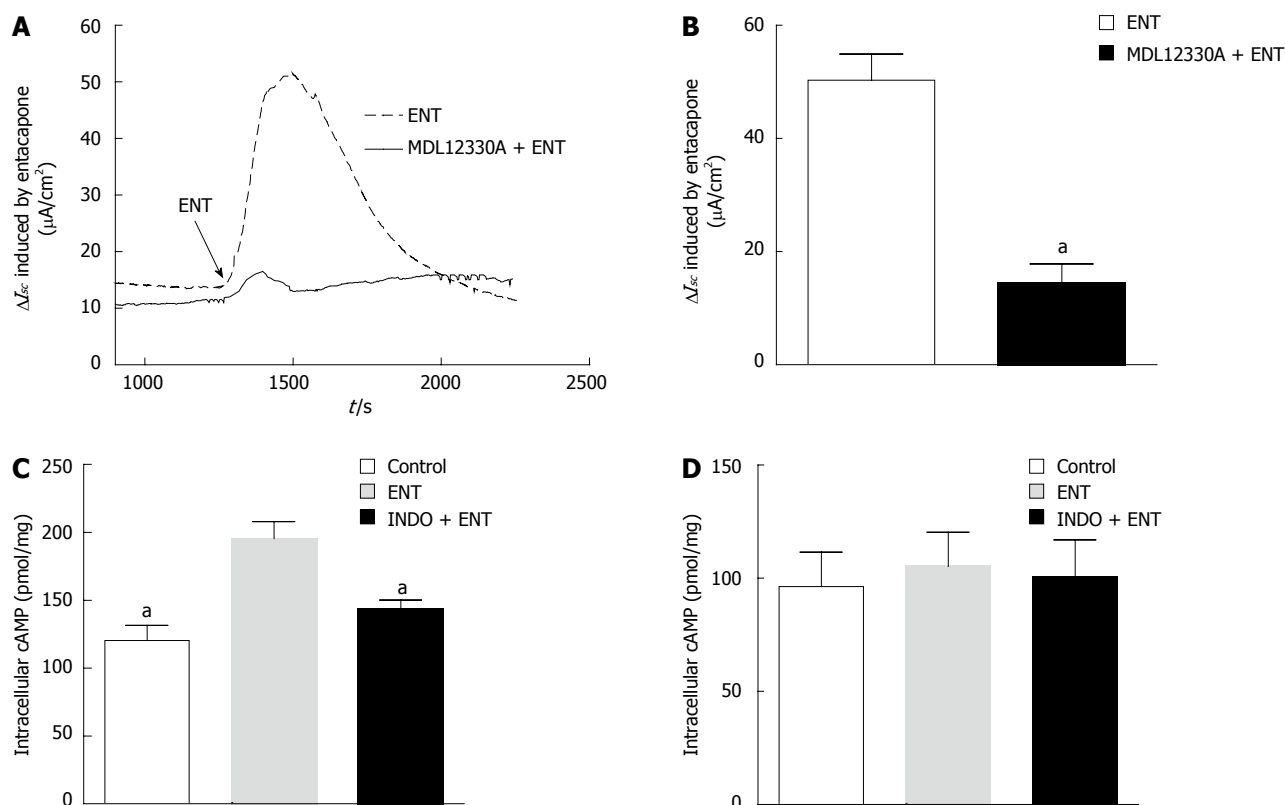


Figure 5 cAMP pathway involved in entacapone-induced Cl⁻ secretion in 6-OHDA Parkinson's disease rats. A: Representation of effects of MDL 12330A (20 μmol/L) on entacapone (ENT)-induced *I*_{sc} changes; B: Generalization of *I*_{sc} caused by ENT after treatment by MDL 12330A; C: Generalization of the roles of indomethacin (INDO) on intracellular cAMP content caused by ENT in 6-OHDA Parkinson's disease (PD) rats; D: Generalization of the roles of INDO on intracellular cGMP content caused by ENT in 6-OHDA PD rats ($n = 6$, $^aP < 0.05$ vs control).

to decrease significantly the entacapone-induced *I*_{sc} ($n = 9$, $P < 0.01$) (Figure 4E and F). Apical application of Ca²⁺-dependent Cl⁻ channel blocker DIDS (200 μmol/L) did not affect entacapone-caused Cl⁻ flux (data not shown).

cAMP-dependent pathway involved in entacapone-induced Cl⁻ secretion in 6-OHDA rat

We know that colon mucosa has mainly Ca²⁺-dependent or cAMP-dependent Cl⁻ channels. cAMP-dependent Cl⁻ channels play an important role in the regulation of mammalian Cl⁻ transport. After the application of 20 μmol/L MMDL-12330A (an adenylate cyclase inhibitor), entacapone-induced Cl⁻ flux was inhibited by 70.69%, from $47.69 \pm 8.91 \mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ to $13.98 \pm 3.42 \mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ (Figure 5A and B, $n = 6$, $P < 0.01$).

Furthermore, a radioimmunoassay was used to detect the amount of cAMP in PD rat colon mucosa (Figure 5C); the basal expression was $132.1 \text{ pmol} \cdot \text{mg}^{-1}$ ($n = 6$). After treatment with 200 μmol/L entacapone, the expression was increased to $181.7 \text{ pmol} \cdot \text{mg}^{-1}$, and indomethacin pretreatment significantly reduced the entacapone-induced cAMP increase. However, with entacapone or indomethacin pre-treatment, the content of cGMP did not obviously change (Figure 5D).

DISCUSSION

As an important metabolic enzyme for neurotransmitters such as dopamine, COMT is not only expressed abundantly in the central nervous system but is also widely expressed in peripheral tissues, such as liver, kidney, stomach, and duodenum^[21]. This study is the first to describe COMT expression in normal and PD rat colon and the characteristics of its localization. This provides histological evidence for further investigation of COMT-related functions.

Currently, clinical studies and application have proven that the COMT inhibitor entacapone has outstanding clinical relevance for the treatment of PD. When entacapone is used in conjunction with L-DOPA, significant improvement is noted in the clinical symptoms^[22,23]. However, gastro-intestinal side effects are also common^[24]. This manuscript utilized *in vitro* colon tissue and investigated the mechanism for entacapone-induced side effects in PD model rats.

The experimental results from this study show that entacapone inhibited colon longitudinal muscle contraction in a dose-dependent manner. This inhibitory effect may be achieved through the β₂ adrenoceptor, subsequently causing the gastro-intestinal smooth muscle of PD patients to contract and relax irregularly, displaying abnormal motility

and obstructing luminal content transport, with a proportion of patients displaying constipation.

I_{sc} recording techniques have been widely utilized to measure epithelial cell electrolyte transport. However, this type of technique lacks chemical selectivity and can only be used to detect electrogenic ion transport^[25,26]. In contrast, SIET is a novel recording method that can overcome the shortcomings of *I_{sc}* recording techniques using specialized ion-sensitive electrodes, such as Cl⁻-sensitive electrodes, to achieve ion flux detection. In this study, in order to make the results more comprehensive, we combined these two methods for determination of ion transport.

In fresh *in vitro* PD rat colon tissue, it was proven that the COMT inhibitor induced colon Cl⁻ secretion. This type of Cl⁻ secretion is mainly electrogenic ion transport mediated by basolateral NKCC and apical membrane Cl⁻ channels, since DPC and bumetanide can block approximately 70% of entacapone-induced Cl⁻ secretion.

The colon mucosa can induce intracellular cAMP expression by exogenous stimulation or endogenous prostaglandin, therefore promoting Cl⁻ secretion^[27,28]. The cyclooxygenase inhibitor indomethacin can block Cl⁻ secretion by 45%, indicating that prostaglandin is involved in PD rat colon mucosa Cl⁻ secretion. In addition, we found that entacapone-induced Cl⁻ secretion in PD rat colons was mainly transduced through the secondary messenger cAMP, because pretreatment with the AC inhibitor MDL-12330A significantly reduced this Cl⁻ secretion and entacapone can induce colon epithelial cell cAMP expression. In the regulation of intestinal water-electrolyte metabolism, the Cl⁻ channel plays an important role. Water also spreads to the enteric cavity due to the osmotic gradient of Cl⁻, causing secretory diarrhea^[29,30]. This may be one of the mechanisms by which entacapone induces diarrhea in PD patients.

In conclusion, this study is the first to prove that COMT is expressed in normal and PD rat colons and that entacapone can inhibit PD rat longitudinal muscle contraction through the β_2 adrenoceptor. It was also discovered that entacapone can induce cAMP-dependent Cl⁻ secretion in PD rats and that endogenous prostaglandin is involved in this process. This study has provided histological evidence for functional studies of COMT in the colon, establishing an experimental basis for the mechanism of entacapone-induced PD gastro-intestinal side effects.

COMMENTS

Background

Catechol-O-methyltransferase (COMT) is expressed in several tissues in humans and rodents. However, the precise localization of COMT in normal and Parkinson's disease (PD) rat intestine is not clear; there are also no studies reporting how entacapone affects ion transport in the intestines of PD patients or whether it affects smooth muscle motility. The effect of entacapone on colon smooth muscle and epithelial ion transport in PD rats was investigated in this study. In addition, the main reasons responsible for entacapone-induced

adverse intestinal effects were explored.

Research frontiers

The authors combined *I_{sc}* recording technique with a novel scanning ion-selective electrode technique method to determine colonic ion transport in PD rats and establish a mechanism of entacapone-induced PD gastro-intestinal side effects.

Innovations and breakthroughs

COMT plays a crucial role in the regulation of dopaminergic systems by catalyzing the inactivation of catecholamines. Entacapone, as a COMT inhibitor, is an emerging drug for PD patients. However, patients experience gastro-intestinal side effects with entacapone treatment; the reasons are unknown. This study was the first to prove that COMT is expressed in normal and PD rat colons and that entacapone can inhibit PD rat longitudinal muscle contraction through the β_2 adrenoceptor. It was also discovered that entacapone can induce cAMP-dependent Cl⁻ secretion in PD rats and that endogenous prostaglandin is involved in this process.

Applications

The study established an experimental basis for the mechanism of entacapone-induced gastro-intestinal side effects with Parkinson's disease.

Terminology

COMT inhibitor refers to a drug that can inhibit catecholamine degradation. Tolcapone and entacapone can be regarded as representative of a new generation of COMT inhibitor. Using a combination of these two drugs with levodopa, the clinical symptoms of patients with Parkinson's disease can be improved remarkably, significantly improving life quality of the patients. However, tolcapone may cause liver damage in individual patients, and entacapone can induce gastro-intestinal side effects in some patients. The further study of related mechanisms is needed to overcome these issues.

Peer-review

The authors have exerted a substantial effort to analyze the effects of entacapone on colon motility and ion transport in Parkinson's disease rats and introduced their findings. This manuscript has a good novel idea. The paper is well organized and written methodology is systematized.

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Basic Study

Primary analysis and screening of microRNAs in gastric cancer side population cells

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Abstract

AIM: To explore the microRNA (miRNA) profiles and to determine the key miRNAs within the side population (SP) cells of the gastric cancer cell line MKN-45.

METHODS: We used fluorescence-activated cell sorting and Hoechst 33342 labeling to obtain SP cells from the human gastric carcinoma cell line MKN-45. The miRNA expression profiles of the SP and major population (MP) cells were examined using a miRNA gene chip, and key miRNAs were obtained according to aberrant expression and the miRNAs' possible targets as predicted by bioinformatics.

RESULTS: Using a significance criterion of a 1.5-fold or greater difference in expression level, we observed an increase in the expression of 34 miRNAs and a decrease in the expression of 34 miRNAs when comparing SP to MP cells. Using quantitative real-time reverse transcription-polymerase chain reaction to test for differentially expressed miRNAs combined with bioinformatics results, we found that the downregulated miRNAs, such as hsa-miR-3175 and hsa-miR-203, and the upregulated miRNAs, including hsa-miR-130a, hsa-miR-324-5p, hsa-miR-34a, and hsa-miR-25-star, may be important in maintaining and regulating the characteristics of SP cells.

CONCLUSION: There are key miRNAs expressed within the SP cells of the gastric cancer cell line MKN-45, and

include hsa-miR-3175, hsa-miR-203, hsa-miR-130a, hsa-miR-324-5p, hsa-miR-34a, and hsa-miR-25-star.

Key words: ATP-binding cassette transporters; Side population cells; Benzimidazoles (Hoe 33342); Stomach neoplasm; Stem cells; MicroRNA

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Core tip: MicroRNAs (miRNAs) act as one mechanism by which coding genes are regulated, and they are involved in many biological and pathological processes including carcinogenesis. However, identifying specific miRNAs is difficult, because many miRNAs possess more than one target and many target genes are regulated by more than one miRNA. We used fluorescence-activated cell sorting and Hoechst 33342 labeling to obtain side population (SP) cells from the human gastric carcinoma cell line MKN-45. The miRNA expression profiles of SP and major population cells were examined by miRNA gene chip analysis, and key miRNAs were identified according to aberrant expression and their possible targets as predicted by bioinformatics.

Zhang HH, Gu GL, Zhang XY, Li FZ, Ding L, Fan Q, Wu R, Shi W, Wang XY, Chen L, Wei XM, Yuan XY. Primary analysis and screening of microRNAs in gastric cancer side population cells. *World J Gastroenterol* 2015; 21(12): 3519-3526 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3519.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3519>

INTRODUCTION

Gastric cancer, with an estimated 986600 new cases worldwide in 2008, is the fourth leading form of cancer and accounts for more than 8% of total cancers^[1]. It is one of the most common and fatal malignancies in East Asia, despite a decreased incidence in the West^[2]. The most effective and specific methods available to treat the disease include local resection, chemotherapy and radiotherapy. However, the question remains regarding whether there are other, more effective and specific methods to treat gastric cancer.

The high mortality rate for gastric cancer is due to increased cases of relapse and metastasis. The cancer stem cell (CSC) hypothesis has received increased attention recently for its convincing explanation for the initiation of relapse and metastasis in several types of carcinomas, including gastric carcinoma^[3-5]. From a large amount of research, several malignant tumor tissues and cell lines have been discovered that possess CSCs, and include acute lymphoblastic leukemia and several solid tumors such as breast, colon, prostate, and gastric cancers^[6-9]. This finding has strengthened the hypothesis that the initiation

of relapse and metastasis may be caused by CSCs. SP cells are a subpopulation of many normal tissues, cancer tissues and cell lines that possess CSC-like phenotypes. However, the exact nature of these cells has yet to be elucidated, particularly regarding the profiles of key miRNAs and their target genes. Our study used fluorescence-activated cell sorting (FACS) to sort side population (SP) cells from gastric cancer cell lines, and then microRNA (miRNA) gene chip analyses were used to examine the miRNA expression profiles of SP and major population (MP) cells. Finally, key miRNAs were obtained according to aberrant expression, and their possible targets were predicted by bioinformatics.

MATERIALS AND METHODS

Cell culture

The human gastric adenocarcinoma cell line MKN-45 was obtained from the Cancer Institute, Chinese Academy of Medical Science and was separately maintained at the Royal Park Memorial Institute (RPMI) in 1640 medium (Invitrogen, United States) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 µg/mL streptomycin. The cells were maintained at 37 °C in a humidified 5% CO₂ incubator.

Fluorescence-activated cell sorting

Nearly confluent MKN-45 cells were harvested by trypsinization with 0.25% Trypsin EDTA (Invitrogen, United States), centrifuged at 1000 r/m for 10 min, washed twice with phosphate buffered saline (PBS), re-suspended at 1×10^6 cells/mL in pre-warmed 37 °C medium of RPMI 1640 with 2% FCS and passed through 40 µm cell strainers (BD Falcon, United States) to obtain single-cell suspensions. The cells were then labeled with Hoechst 33342 (Sigma-Aldrich, United States) at a concentration of 5 µg/mL, and the labeled cells were incubated in the dark for 60-75 min in a 37 °C water bath with intermittent mixing, with or without 75 µmol/L verapamil (Sigma-Aldrich, United States). The cells were suspended in ice-cold PBS containing 2% FBS after staining, and maintained at 4 °C until flow cytometry analysis. Cells were labeled with 1 µg/mL propidium iodide (PI) to assess viability 5 min before examination. The stained cells were analyzed using a FACS Aria II (BD Biosciences, San Jose, CA, United States). The Hoechst dye was excited by an ultraviolet laser at 375 nm, and its fluorescence measured with 450/40 nm (Hoechst blue) and 695/40 LP (long-pass, Hoechst red) optical filters.

miRNA microarray assay and quantitative real-time quantitative reverse transcription-polymerase chain reaction

We used the Affymetrix gene chip miRNA 2.0 array according to the manufacturer's instructions for our

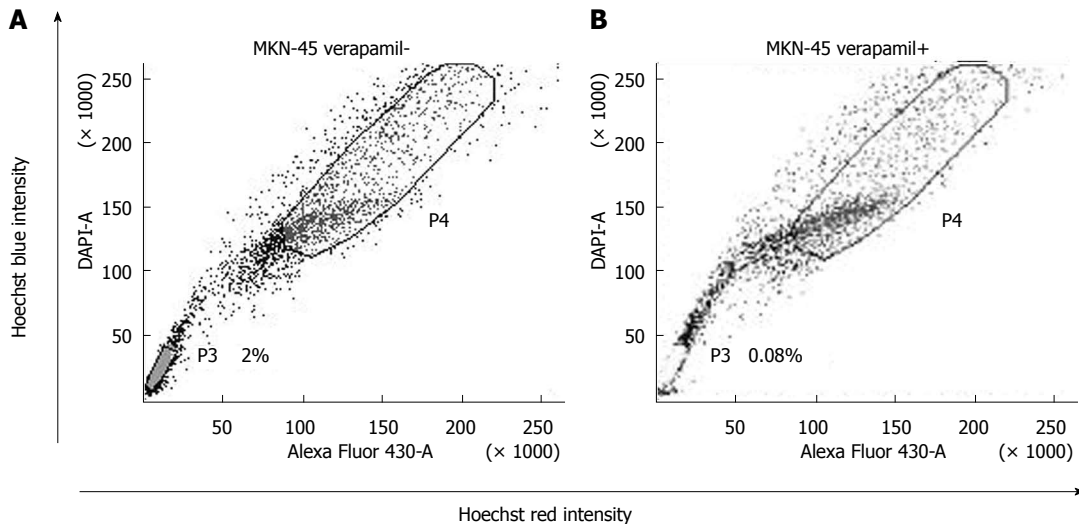


Figure 1 Side population cell analysis. The P3 gate was the side population (SP) cells and the P4 gate was the major population (MP) cells. A: SP ratio in MKN-45 was 2.0%; B: The SP cells obviously decreased with both Hoechst 33342 and verapamil. Figure 1 cited partly from the article of Not All Side Population Cells Contain Cancer Stem-Like Cells in Human Gastric Cancer Cell Lines which published in *Dig Dis Sci* 2013; 58: 132-139.

miRNA microarray assay (Affymetrix Int, CA, United States). Each miRNA microarray chip contained 1100 identified human miRNAs probes. Briefly, total RNA (200 ng) was polyadenylated and tagged with biotin HSR ligation, reverse transcribed, and the cDNA was hybridized to a miRNA bead chip for 16 h at 48 °C. The hybridized miRNA bead chip was washed for 1 h in a washing station, and the arrays were scanned on an Affymetrix reader. Microarray data for each sample were normalized to the median, and the processing and analysis was performed with Affymetrix software.

Total RNA, including miRNA, was extracted from MP and SP cells using Trizol reagent according to the manufacturer's instructions. SYBR green mRNA quantitative real-time polymerase chain reaction (PCR) was performed using the sequence-specific stem-loop primers supplied by RiboBio (Guangzhou, China). Quantitative reverse transcription PCR (RT-PCR) was conducted using a standard SYBR Green PCR kit (QIAGEN) protocol with a Light Cycler 480 real-time instrument (Roche). The relative expression was calculated using the $2^{-\Delta\text{CT}}$ method. The transcription levels of U6 were used as an internal control.

Bioinformatic analysis

In this study, predicted targets of novel miRNA were analyzed and determined using three publicly available algorithms including MiRanda (<http://www.microrna.org>), PicTar (<http://pictar.mdc-berlin.de>) and TargetScan (<http://www.targetscan.org/>). These searchable websites predict biological targets of miRNAs by searching for the presence of conserved 8-mer and 7-mer sites that match the seed region of the miRNA and provide details of the 3'-UTR alignments with predicted sites. To decrease the number of false-positive results, only putative target genes predicted by at least two programs were accepted. In addition, we used a Capital-Bio Molecule

Annotation System Version 3.0 to perform gene ontology analysis on the target genes and the specific biological process categories that were enriched.

RESULTS

Gastric cancer cell MKN-45 contain SP cells

Through the use of FACS, we determined that the gastric cancer cell line MKN-45 contained SP cells. We set the sorting gate according to the ability of cells to efflux Hoechst 33342 and their sensitivity to verapamil. The lower left quadrant of the FACS profile, which showed Hoechst blue and could be blocked by verapamil, was defined as the SP. The top right quadrant of the FACS profile, which showed Hoechst red and was not blocked by verapamil, was defined as the MP. We targeted these cells and differentiated them for further study. In the MKN-45 cells, the percentage of SP was 2.0% of the total cells (Figure 1A).

miRNA expression profiles and quantitative real-time RT-PCR

To investigate whether miRNAs were differentially expressed in SP and MP cells, we compared their miRNA expression profiles using a miRNA microarray (Figure 2A and B). Using a significance criterion of a 1.5-fold or greater difference in expression level, we observed the increased expression of 34 miRNAs and the decreased expression of 34 miRNAs in SP vs MP cells. The top 15 upregulated and 11 downregulated miRNAs are shown in Table 1. Next, we used quantitative real-time RT-PCR to test the differentially expressed miRNAs, and we found that downregulated miRNAs, such as hsa-miR-3175 and hsa-miR-203, and upregulated miRNA, including hsa-miR-130a, hsa-miR-324-5p, hsa-miR-34a, and hsa-miR-25-star, may be important in the maintenance and regulation of SP cell characteristics.

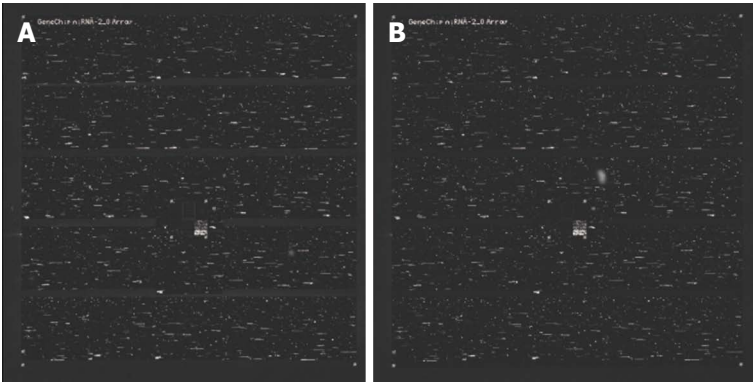


Figure 2 MicroRNA microarray. Affymetrix genechip 2.0 was used to examine microRNA expression profiles of SP and MP cells of MKN-45. A: Hybridized genechip with microRNA of SP cells; B: Hybridized genechip with microRNA of MP cells. SP: Side population; MP: Major population.

Table 1 MicroRNA microarray and quantitative real-time-polymerase chain reaction analyses results							
Down-regulated genes	Fold change microarray	Δ CT	$-\Delta\Delta$ CT	Upregulated genes	Fold change microarray	Δ CT	$-\Delta\Delta$ CT
		qRT-PCR				qRT-PCR	
hsa-miR-3175	7.29	21.82	-1.32	hsa-miR-25-star	27.35	6.63	4.47
hsa-miR-1246	3.46	14.07	-0.64	hsa-miR-1275	5.13	13.77	0.48
hsa-miR-663	3.12	10.69	-0.13	hsa-miR-339-5p	4.03	15.63	0.89
hsa-miR-1281	2.93	14.72	-0.74	hsa-miR-140-3 p	3.56	13.30	0.41
hsa-miR-422a	2.56	14.41	-0.69	hsa-miR-197	3.36	14.08	0.63
hsa-miR-1228-star	2.41	16.62	-0.98	hsa-miR-362-5p	3.01	15.25	0.86
hsa-miR-1975	2.10	12.84	-0.38	hsa-miR-324-5p	2.75	17.85	1.06
hsa-miR-1826	2.09	14.18	-0.67	hsa-miR-34a	2.70	23.46	2.32
hsa-miR-1915	2.08	10.83	-0.14	hsa-miR-210	2.17	13.30	0.44
hsa-miR-4284	2.07	14.34	-0.65	hsa-miR-500-star	2.06	11.06	0.18
hsa-miR-203	1.57	26.13	-3.84	hsa-miR-130a	1.77	22.49	2.13
				hsa-miR-130b	1.61	17.34	0.85
				hsa-miR-18a	1.60	13.41	0.46
				hsa-miR-29a	1.57	15.46	0.80
				hsa-miR-149	1.54	11.96	0.32

Differentially expressed microRNAs (miRNAs) in SP and MP cells of the gastric cancer cell line MKN-45 as determined by miRNA microarray and quantitative real-time-PCR analyses. These results showed that the miRNA profiles of SP cells differed from the miRNA profiles of MP cells. Moreover, downregulated miRNAs, such as hsa-miR-3175 and hsa-miR-203, and upregulated miRNAs, including hsa-miR-130a, hsa-miR-324-5p, hsa-miR-34a, and hsa-miR-25-star, showed significant differences in expression between SP and MP cells when tested by quantitative real-time PCR. SP: Side population; MP: Major population; PCR: Polymerase chain reaction.

Bioinformatics analysis

There are 33036 miRNA-target relationships predicted by at least two of the three database software programs MiRanda, TargetScan and Pictar^[10-12]. However, there are 469 miRNA-target relationships after experimental verification using the Tarbase database. There are 9 microRNAs with predicted or experimentally verified targets among 68 differentially expressed microRNAs, including 8 upregulated microRNAs and one downregulated microRNA. Hsa-miR-130a shows the largest number of target genes, 406, followed by hsa-miR-29a, at 402. These 9 microRNAs have 2006 targets that have been predicted or experimentally validated (Table 2). The targets are often regulated by more than one miRNA (Table 3). Finally, we can construct a global microRNA regulatory network by integrating the human protein-protein interaction data with the predicted microRNA-target relationships. This network is closely linked with the module function analysis^[13,14] (Figure 3).

DISCUSSION

The increased mortality rate for gastric cancer is due to increased cases of relapse and metastasis. Increasing numbers of studies have shown that CSCs are involved in tumor progression and metastasis and are associated with increased aggressiveness and metastasis *in vivo* but not *in vitro*^[15]. Several published reports have demonstrated that SP cells are being increasingly used as an effective method to obtain and identify stem cells or putative CSCs^[6-9]. In our study, we obtained gastric CSCs from MKN-45 cells through sorting by FACS technology, and characterized their CSC properties^[16]. Thus, we used SP cells as a gastric CSC model to elucidate their miRNA expression profiles, predict miRNA targets, and analyze possible miRNA modulating mechanisms.

We determined the miRNA expression profiles of SP and MP cells sorted from the MKN-45 cell line using a miRNA microarray, which showed that the

Table 2 Differentially expressed microRNAs and their target number and known targets

microRNA	Sp/Mp (FC)	Target number	Validated target number	Known targets
hsa-miR-130a	1.77	406	3	MEOX2; TAC1; HOXA5
hsa-miR-29a	1.57	402	3	DNMT3A; BACE1; DNMT3B
hsa-miR-130b	1.61	392	0	
hsa-miR-203	0.64	275	0	
hsa-miR-34a	2.70	260	3	E2F3; CCND1; CDK6
hsa-miR-149	1.54	129	0	
hsa-miR-18a	1.60	104	0	
hsa-miR-324-5p	2.75	33	0	
hsa-miR-210	2.17	5	1	EFNA3

Bioinformatic analysis of differentially expressed microRNAs indicated that differentially expressed microRNAs had predicted or experimentally verified target genes. Moreover, there are multiple targets for many miRNAs, but most of these targets have not been studied or validated.

Table 3 Genes that are regulated by more than 4 microRNAs

Gene ID	Gene name	microRNA number	All microRNAs
80218	NAA50	5	hsa-miR-203; hsa-miR-18a; hsa-miR-34a; hsa-miR-130a; hsa-miR-130b
164	APIG1	5	hsa-miR-324-5p; hsa-miR-130b; hsa-miR-29a; hsa-miR-130a; hsa-miR-203
7803	PTP4A1	4	hsa-miR-203; hsa-miR-130a; hsa-miR-130b; hsa-miR-29a
90355	C5orf30	4	hsa-miR-130b; hsa-miR-18a; hsa-miR-130a; hsa-miR-203
80829	ZFP91	4	hsa-miR-130b; hsa-miR-130a; hsa-miR-324-5p; hsa-miR-29a
28514	DLL1	4	hsa-miR-149; hsa-miR-130a; hsa-miR-130b; hsa-miR-34a
23013	SPEN	4	hsa-miR-203; hsa-miR-130a; hsa-miR-29a; hsa-miR-130b
57659	ZBTB4	4	hsa-miR-18a; hsa-miR-149; hsa-miR-130a; hsa-miR-130b
23261	CAMTA1	4	hsa-miR-34a; hsa-miR-203; hsa-miR-130b; hsa-miR-130a
9444	QKI	4	hsa-miR-18a; hsa-miR-130b; hsa-miR-29a; hsa-miR-130a
166336	PRICKLE2	4	hsa-miR-130b; hsa-miR-29a; hsa-miR-130a; hsa-miR-203
5156	PDGFRA	4	hsa-miR-130a; hsa-miR-149; hsa-miR-130b; hsa-miR-34a

Predicted target genes of key microRNAs. Some microRNAs, such as hsa-miR-324-5p, hsa-miR-130b, hsa-miR-29a, hsa-miR-130a, and hsa-miR-203, modulate multiple genes; these results indicate that differentially expressed microRNAs and their targets have a reticular network.

miRNA profiles were significantly different between SP and MP cells; the total number of differentially expressed miRNAs was 68. Moreover, we tested the differentially expressed miRNAs using quantitative real-time RT-PCR, and found that downregulated miRNAs, such as hsa-miR-3175 and hsa-miR-203, and upregulated miRNAs, including hsa-miR-130a, hsa-miR-324-5p, hsa-miR-34a, and hsa-miR-25-star, showed significant differences between SP and MP cells. In general, miRNAs upregulated in SP cells act as oncogenic miRNAs, while downregulated miRNAs act as tumor suppressors. As a tumor suppressor, hsa-miR-3175 appears to be a novel biomarker; there have been limited studies of this miRNA, and further research to validate whether it is involved in the maintenance of cancer cell stemness is warranted. Conversely, hsa-miR-203 appears to be involved in maintaining the stemness of cancer cells rather than in tumorigenicity, and it plays a crucial role in the progression of human carcinoma. Decreased expression of miR-203 was significantly related to poor differentiation, advanced clinical stage, T3-4 tumor grade, lymph node metastasis, and decreased 5-year overall survival. Furthermore, miR-203 may regulate the expression of E-cadherin mesenchymal transition, and CD44, a marker of CSCs^[17], suggesting that

miR-203 could be involved in the regulation of CSCs. Interestingly, another study had found that miR-203 was downregulated and Bmi-1 was upregulated in the SP cells of the esophageal squamous cell carcinoma cell line EC9706. MiR-203 over-expressing cells also showed a significant reduction in colony formation that was resistant to chemotherapeutic drug treatment, and tumorigenicity in nude mice. These results indicate that the stem renewal factor Bmi-1 is a direct target of miR-203. The regulation of Bmi-1 by miR-203 may play an important role in controlling the proliferation and self-renewal of esophageal cancer stem-like cells^[18]. Our study on miR-203 in SP cells which showed downregulated expression, is in agreement with the above results. However, there is a lack of consensus in the literature about this finding. Stánitz *et al.*^[19] reported contradictory findings, in which gastric adenocarcinoma patients with regular alcohol consumption showed an upregulation of miR-203, miR-205, and miR-223^[19]. Therefore, we cannot state conclusively that miR-203 acts as a tumor suppressor while maintaining stemness, such as proliferation, self-renewal, and tumorigenicity, of gastric CSCs. In the future, we will continue to study the specific mechanism of action of miR-203 in gastric cancer. As for other oncogenic miRNAs identified in

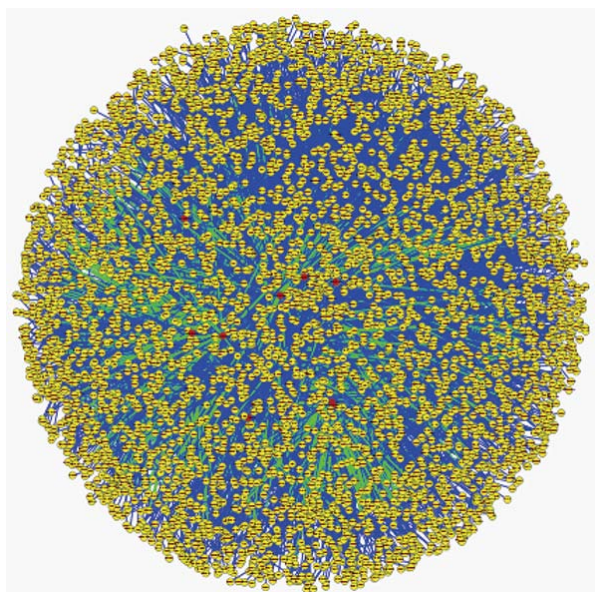


Figure 3 Visualization of the global microRNA regulated network. Red nodes represent microRNA, yellow nodes represent coding genes. Green edge represents the microRNA-target relationship, blue edge represents the protein-protein interaction.

our study, hsa-miR-130a, hsa-miR-324-5p, hsa-miR-34a, and hsa-miR-25-star, are known not to play roles in many biological processes aside from maintaining stemness of CSCs. The results of a previous study show that the low miR-34a expression levels in many cells together with their colony forming efficiency, a characteristic property of CSCs, can be inhibited by miR-34a replacement or synthetic miR-34a *in vivo*. This finding is in contrast with our study results on miR-34a *in vitro*. Furthermore, the expression of miR-34a antagomirs in these cells promoted tumor development, indicating that miR-34a is a negative regulator of the tumorigenic properties of cancer cells and the stemness characteristics of CSCs^[20,21]. A further study has found that there is a novel regulatory network composed of p53, CD95, let-7, and miR-34a that affects cancer cell survival, differentiation, and sensitivity to apoptotic signals^[22]. Moreover, the treatment of pancreatic CSCs with chromatin-modulating agents results in the inhibition of Bcl-2, CDK6 and SIRT1, which are the putative targets of miR-34a. MiR-34a upregulation by these agents also induced acetylated p53, p21 (WAF1), p27 (KIP1) and PUMA (BCL2 binding component 3) in pancreatic CSCs^[23]. From these results, we can conclude that miR-34a acts as a major tumor suppressor in maintaining the stemness of CSCs, but the putative targets, potential functions and mechanism of miR-34a in gastric cancer SP cells remain to be explored and detailed in further studies. Another oncogenic miRNA is miR-130a, which showed high levels of expression in some cancer cells^[24], especially in some drug-resistant cell lines^[25], and low levels in other cancer cells^[26,27]. Interestingly, our results on miR-130a showed high

levels in SP cells. More information about miR-130a and CSCs, the putative targets regulated by miR-130a, and the mechanism of miR-130a in gastric CSCs requires further study. miR-324-5p and miR-25-star are 2 additional oncogenic miRNAs that were identified in our study. miR-324-5p can inhibit the proliferation of glioma cells *via* the targeted regulation of glioma-associated oncogene 1^[28]. It is also involved in the development of cervical cancer as a result of human papillomavirus infection, which might regulate the oncogene E5. Whether miR-324-5p participates in the regulation of gastric CSCs has yet to be studied. MiR-25-star may be a new player in the behavior of cancer and CSCs, but there is nothing in the literature to date.

A further bioinformatic analysis found that hsa-miR-130a, hsa-miR-29a, hsa-miR-210, and hsa-miR-34a each had more than one predicted or experimentally verified targets, while other miRNAs, such as hsa-miR-203 and hsa-miR-324-5p, had no known predicted or experimentally verified targets (Table 2). However, the latter miRNAs often regulated a common target at the same time (Table 3), such as putative transcription factors, including Dll1 (Delta-like 1), ZBTB4, (the Zinc fingers, C2H2 and BTB domain containing (ZBTB) family member), CAMTA1 (calmodulin-binding transcription activator 1), QKI (the RNA-binding protein Quaking) and PDGFR α (platelet-derived growth factor receptor alpha) among others. The transcription factor Dll1, which is one of the Notch signaling pathway ligands, is involved in the maintenance of stem cells during embryogenesis and in self-renewing tissues of the adult. The epigenetic regulation of the Notch ligand DLL1 controls Notch1 signaling activation in gastric cancer, and Notch1 inhibition is associated with the diffuse type of gastric cancer^[29]. CAMTA1 is a putative transcription factor in glioblastoma stem cells that acts as a tumor suppressor gene and is a target of miR-9 and miR-17^[30]. QKI is a newly identified tumor suppressor found in multiple cancers whose expression is significantly decreased in most GC tissues; the reduced QKI expression correlates well with poor differentiation status, depth of invasion, gastric lymph node metastasis, distant metastasis, advanced TNM stage, and poor survival^[31]. In mouse and human CRC cells, miR-574-5p has been shown to regulate QKI isoforms post-transcriptionally and to cause altered β -catenin and p27 (Kip1) expression, increased proliferation, migration and invasion and decreased differentiation and cell cycle exit^[32]. PDGFR α is involved in the resistance of the stem cell factor kit to imatinib mesylate in gastrointestinal stromal tumors^[33]. Deficiencies in the downregulation of PDGFR α have been identified as a candidate mechanism for tumor cell proliferation, and the knockdown of PDGFR α by siRNA results in a reduction in cell growth^[34]. Other targets, such as E2F3, CCND1 and CDK6, are also important genes that may be involved in tumorigenicity and the maintenance of stemness in CSCs. Therefore, according to our results, differentially expressed

microRNAs and their targets have a reticular network. In fact, the maintenance and modulation of stemness in CSCs may be the result of regulation by a double shift network consisting of 2 groups of miRNAs and their target genes working with opposing functions. This work on miRNAs and their possible targets lays the groundwork for future studies in this area.

In summary, we elucidated the expression profiles of microRNAs using SP cells as a gastric CSC model and predicted the key microRNA targets by bioinformatics analysis. These findings will pave the way for further studies on the molecular modulation of cancer mechanisms by microRNA.

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COMMENTS

Background

Gastric cancer remains the most common cancer worldwide. The most effective and specific methods available to address the disease include local resection, chemotherapy, and radiotherapy. However, the question remains as to whether there are any other more effective and specific methods to treat gastric cancer. The cancer stem cell (CSC) hypothesis has garnered more attention in recent times for its convincing explanation for the initiation of relapse and metastasis of cancers including gastric carcinoma. Side population (SP) cells are a subpopulation found in many normal tissues, cancer tissues, and cell lines that possess CSC-like phenotypes. However, the exact nature of these cells has yet to be elucidated, especially regarding key microRNA (miRNA) profiles and the target genes regulated by these miRNAs.

Research frontiers

MiRNAs act as one mechanism underlying the regulation coding genes and are involved in many biological and pathological processes including carcinogenesis. Increasingly, aberrant miRNA expression has been found in tumor tissues or cells; however, there has been little research on the miRNAs of CSCs or in SP cells that possess CSC-like characteristics.

Innovations and breakthroughs

This study is the first to use SP cells from the gastric cancer cell line MKN-45 as a CSC model to elucidate the miRNAs profiles, and we discovered key miRNAs within these SP cells. This study provides the groundwork for additional research, and the results of this study provide mechanisms for future miRNA studies.

Applications

Previous studies have investigated the diagnostic and prognostic value of miRNAs in gastric cancer. One such study identified a 7-miRNA signature (miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p and miR-126) that is an independent predictor for overall survival and relapse-free survival shown by multivariate analysis. However, the most specific and effective miRNAs have yet to be discovered. The association of miRNA deregulation with the pathogenesis and progression of malignant disease illustrates the great potential of utilizing miRNAs as targets for therapeutic interventions. However, the therapeutic potential of miRNA-based treatments in malignant disease remains largely unexplored.

Terminology

Fluorescence activated cell sorting (FACS) is a technique based on nucleic acid dye Hoechst 33342 efflux, which can sort cells into SP cells and MP cells. The CSC hypothesis suggests that tumors consist of tumor-forming, self-renewing CSCs within a large population of non-tumor-forming cancer cells. CSCs resist standard chemotherapy that reduces tumor mass by killing non-stem cells. During remission, CSCs can regenerate all the cell types in the tumor through their stem cell-like behavior, resulting in relapse of the disease. The side

population is a subpopulation of many normal tissues, cancer tissues and cell lines that possess CSC-like phenotypes. They can be isolated by fluorescence-activated cell sorting techniques.

Peer-review

This is an interesting manuscript. The study is well designed. In this study, the authors find that the gastric cancer cell line MKN-45 contains SP cells that possess cancer stem-like characteristics, and further bioinformatic analysis found that there are key miRNAs in the cancer stem cell-like SP cells of the gastric cancer cell line MKN-45. However, the specificity and effectiveness of the miRNAs and their diagnostic and prognostic values will require additional studies to test and prove.

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Basic Study

High persistence rate of hepatitis B virus in a hydrodynamic injection-based transfection model in C3H/HeN mice

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Abstract

AIM: To optimize the viral persistence rate in a hydrodynamic injection (HI) based hepatitis B virus (HBV) transfection mouse model.

METHODS: (1) 5-6-wk-old male C3H/HeN and C57BL/6 mice were hydrodynamically injected with 10 μ g endotoxin-free pAAV/HBV1.2 plasmid DNA *via* the tail vein. Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and HBV DNA, both in the serum and liver, were detected at different time points post HI by ELISA, immunohistochemical staining or quantitative polymerase chain reaction (PCR); (2) male C3H/HeN and C57BL/6 mice, either hydrodynamically injected mice at 10 wk post HI or naïve mice, were all immunized subcutaneously with 5 μ g HBsAg formulated in complete Freund's adjuvant three times at a 2-wk interval. Two weeks after the final immunization, splenocytes were isolated for T cell function analysis by ELISPOT assay; and (3) five weeks post HI, C3H/HeN mice were intragastrically administered 0.1 mg/kg entecavir once a day for 14 d, or were intraperitoneally injected with 1 mg/kg interferon (IFN)- α twice a week for 2 wk, or were treated with PBS as controls. The sera were collected and assayed for HBV DNA on days 0, 7 and 14 after drug treatment.

RESULTS: (1) Approximately 90% (22/25) of the injected C3H/HeN mice were still HBsAg-positive at 46 wk post HI, whereas HBsAg in C57BL/6 mice were completely cleared at 24 wk. Serum levels of HBeAg in C3H/HeN mice were higher than those in C57BL/6 mice from 4 wk to 46 wk. HBV DNA levels in the hydrodynamically injected C3H/HeN mice were higher than those in the C57BL/6 mice, both in the serum

(from 4 wk to 46 wk) and in the liver (detected at 8 wk and 46 wk post HI). Histology showed that hepatitis B core antigen and HBsAg were expressed longer in the liver of C3H/HeN mice than in C57BL/6; (2) HBsAg specific T cell responses after HBsAg vaccination in hydrodynamically injected C3H/HeN and C57BL/6 mice, or naive control mice were detected by ELISPOT assay. After stimulation with HBsAg, the frequencies of IFN- γ producing splenocytes in the hydrodynamically injected C3H/HeN mice were significantly lower than those in hydrodynamically injected C57BL/6 mice, control C3H/HeN and control C57BL/6 mice, which were 0 , 17 ± 7 , 18 ± 10 , and 41 ± 10 SFCs/ 10^6 splenocytes, respectively, and the mean spot sizes showed the same pattern. Even just stimulated with PMA and ionomycin, T-cell responses elicited in the vaccinated control C3H/HeN were much higher than those in hydrodynamically injected C3H/HeN mice; and (3) For drug treatment experiments on the hydrodynamically injected C3H/HeN mice, serum HBV DNA levels in the entecavir treatment group declined (131.2 folds, $P < 0.01$) on day 7 after treatment and kept going down. In the group of IFN- α treatment, serum HBV DNA levels declined to a lowest point (6.42 folds, $P < 0.05$) on 7 d after treatment and then rebounded.

CONCLUSION: We have developed a novel HI-based HBV transfection model using C3H/HeN mice, which had a higher HBV persistence rate than the classic C57BL/6 mouse model.

Key words: Hepatitis B virus; Hydrodynamic injection; Viral persistence; Liver; Mouse

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Core tip: In the classic hepatitis B virus (HBV) hydrodynamic injection (HI) model using C57BL/6 mice, only about 30% of the injected mice carried HBV for more than 12 wk. Here we injected the pAAV-HBV1.2 plasmid into C3H/HeN mice and observed that the hepatitis B surface antigen, hepatitis B e antigen and viral DNA persisted even up to 46 wk in about 90% of the hydrodynamically injected mice. Applying interferon- α or entecavir in this HI model decreased HBV DNA *in vivo*. Hence, C3H/HeN is a suitable mouse strain for the persistent HBV HI model, which might be useful for chronic hepatitis B research and therapeutic drug development.

Peng XH, Ren XN, Chen LX, Shi BS, Xu CH, Fang Z, Liu X, Chen JL, Zhang XN, Hu YW, Zhou XH. High persistence rate of hepatitis B virus in a hydrodynamic injection-based transfection model in C3H/HeN mice. *World J Gastroenterol* 2015; 21(12): 3527-3536 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3527.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3527>

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) in the human liver remains a major health problem globally^[1]. More than 400 million people have been infected and about one million patients die annually from HBV infection^[2]. Currently, interferon (IFN)- α and nucleoside/nucleotide analogs have been mainly used for clinical treatment of chronic hepatitis B (CHB). Although nucleoside/nucleotide analogs inhibit HBV replication, drug resistance remains an unsettled tough issue in clinical practice^[3]. On the other hand, IFN- α enhances host immune responses and promotes HBV clearance. However, only 30% of CHB patients showed a sustained response to IFN- α treatment, which limits the clinical effect and application of IFN- α ^[4]. New therapeutic strategies are needed to be developed to improve the treatment of CHB.

It is believed that the balance between viral replication and the host immune response during chronic HBV infection determines the pathogenesis and outcomes of CHB. Nevertheless, the etiological mechanisms of the host immune responses that lead to HBV persistence are still to be elucidated completely yet, though some components of the immune system, particularly cellular immune responses, have shown to be involved in the clearance of HBV^[5].

However, the research of HBV infection, either studies on immunological mechanisms or therapeutic drug development, has been hampered by the shortage of suitable animal models^[6]. Compared with chimpanzees, woodchuck and duck, mouse is the ideal laboratory animal for its convenient availability, easy husbandry and low cost, and most importantly, their well characterized genetic background, techniques for genetic modification, and abundance of immunological reagents. Despite the inability of HBV to propagate in mouse, several mouse models of HBV have been developed, including HBV transgenic mice, adenovirus or adenovirus-associated-virus (AAV) based HBV transduction models, and hydrodynamic injection (HI) based HBV transfection models^[6].

Introducing the HBV genome into the mouse liver by HI *via* the tail vein represents a model that mimics the natural course of chronic HBV infection in human without side effects from the viral vectors, such as immune responses against adenovirus^[7]. Nonetheless, in the classic model developed by HI of an HBV plasmid into C57BL/6 mice, only less than 20%-30% injected mice carried HBV for 24 wk^[8]. Recently we hydrodynamically injected the HBV plasmid into C3H/HeN mice and succeeded in delaying the mouse immune clearance of HBV. About 90% the injected C3H/HeN mice maintained HBV persistence even up to 46 wk post HI. Applying IFN- α and entecavir in this model led to HBV DNA decrease *in vivo*. Thus, this

novel HI based model of HBV might provide a more stable platform for the research of HBV persistence infection.

MATERIALS AND METHODS

Ethics statement

All procedures on mouse were reviewed by the Institutional Animal Care and Use Committee of Shanghai Public Health Clinical Center and were performed in strict accordance with the approved protocol.

Preparation of an HI based mouse model with a recombinant HBV plasmid

The replication-competent recombinant HBV plasmid pAAV/HBV1.2 was kindly provided by Prof. Peijer Chen, National Taiwan University College of Medicine. Specific pathogen free (SPF) C3H/HeN and C57BL/6 mice were purchased from the animal facilities of Shanghai Public Health Clinical Center. Male C3H/HeN and C57BL/6 mice (5–6-wk-old) were injected with 10 µg endotoxin-free pAAV/HBV1.2 plasmid DNA into the tail vein in a volume of PBS equivalent to 10% of the mouse body weight and the total volume was delivered within 5 s, which was so-called HI as previously described^[9,10]. The serum specimens were assayed for hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) or HBV DNA at the indicated time points after injection. When the mice were sacrificed, the spleens were collected for T cell function analysis and the livers were collected and preserved in 4% PFA (paraformaldehyde) for immunohistochemical analysis.

Detection of HBV antigen

Serum specimens were collected and assayed for HBsAg and HBeAg at 0, 1, 2, 3, 4, 5, 8, 10, 16, 24 and 46 wk after HI of pAAV/HBV1.2. Serum levels of HBsAg were determined with an ELISA kit (Kehua, Shanghai, China). The levels of HBeAg were determined by ELISA (Kehua, Shanghai, China).

Detection of serum HBV DNA

Serum samples were collected at 0, 1, 2, 3, 4, 5, 8, 12, 16, 24 and 46 wk after HI of pAAV/HBV1.2. For HBV DNA extraction, 10 µL mouse serum was added into 40 µL PBS, and digested with 10 µg DNaseI for 1 h at 37 °C. Then, 100 µL lysis buffer (20 mmol/L Tris-HCl, 20 mmol/L EDTA, 50 mmol/L NaCl, and 0.5% SDS) containing 50 mg proteinase K was added. After incubation at 65 °C overnight, viral DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was rinsed with 70% ethanol and resuspended in 10 µL ddH₂O. The quantification of HBV DNA was performed using a routine real-time PCR procedure described previously, with a SYBR Green Real-time PCR Master Mix kit (TOYOBO, Osaka, Japan)^[11].

Detection of liver HBV DNA

Liver tissues were collected from mice receiving HI killed at 46 wk. The total DNA of the liver was extracted as described above and detected for HBV DNA by real-time PCR or DpnI enzyme before real-time PCR.

Immunohistochemistry

Liver samples were collected 46 wk post HI. Intrahepatic HBsAg was visualized by immunohistochemical staining of tissues incubated with mouse anti-HBs antibody (Maixin.Biotech, Fuzhou, China), rabbit anti-HBc antibody (Maixin.Bio city, Fuzhou, China), and HRP (Maixin.Bio city, Fuzhou, China).

Mouse vaccination

Male C3H/HeN and C57BL/6 mice at 10 wk after HI with 10 µg pAAV/HBV1.2 plasmid or naïve control male C3H/HeN and C57BL/6 mice were all immunized subcutaneously with 5 µg HBsAg formulated in complete Freund's adjuvant (CFA) three times at a 2-wk interval. Mice were euthanized 2 wk after the final immunization and fresh splenocytes were collected.

IFN-γ ELISPOT assay

Freshly isolated mouse splenocytes were adjusted to a density of 4×10^6 cells/mL and plated into 96-well ELISPOT plates (BD Bioscience, Franklin Lakes, New Jersey, United States) coated with an anti-mouse IFN-γ antibody at 50 µL/well (2×10^5 cells per well). The splenocytes were stimulated with 10 µg/mL HBsAg protein. After incubation at 37 °C with 5% CO₂ for 20 h, the ELISPOT plates were developed according to the manufacturer's manual and read with Immunospot Reader (ChamspotIII, Beijing Sage Creation Science, China).

IFN-α or entecavir treatment assay

An HI based mouse model of HBV was established as described above in fifteen 4–6-wk-old male C3H/HeN mice. Five weeks post HI, the mice were randomly divided into three groups (five mice each group). The first group was intragastrically administered 0.1 mg/kg entecavir (Baraclude, Bristol-Myers Squibb, New York, United States) once a day for 14 d; the second group was intraperitoneally injected with 1 mg/kg interferon-α (IFN-α, R and D, R and D Systems, Minneapolis, United States) twice a week for 2 wk; the third group was treated with PBS as a control. Serum specimens were collected and assayed for HBV DNA on days 0, 7 and 14 after treatment.

Statistical analysis

Comparisons between two groups were performed by unpaired *t*-test and comparisons among three or more groups were performed using one-way analysis of variance (GraphPad Software, Inc.). Significant

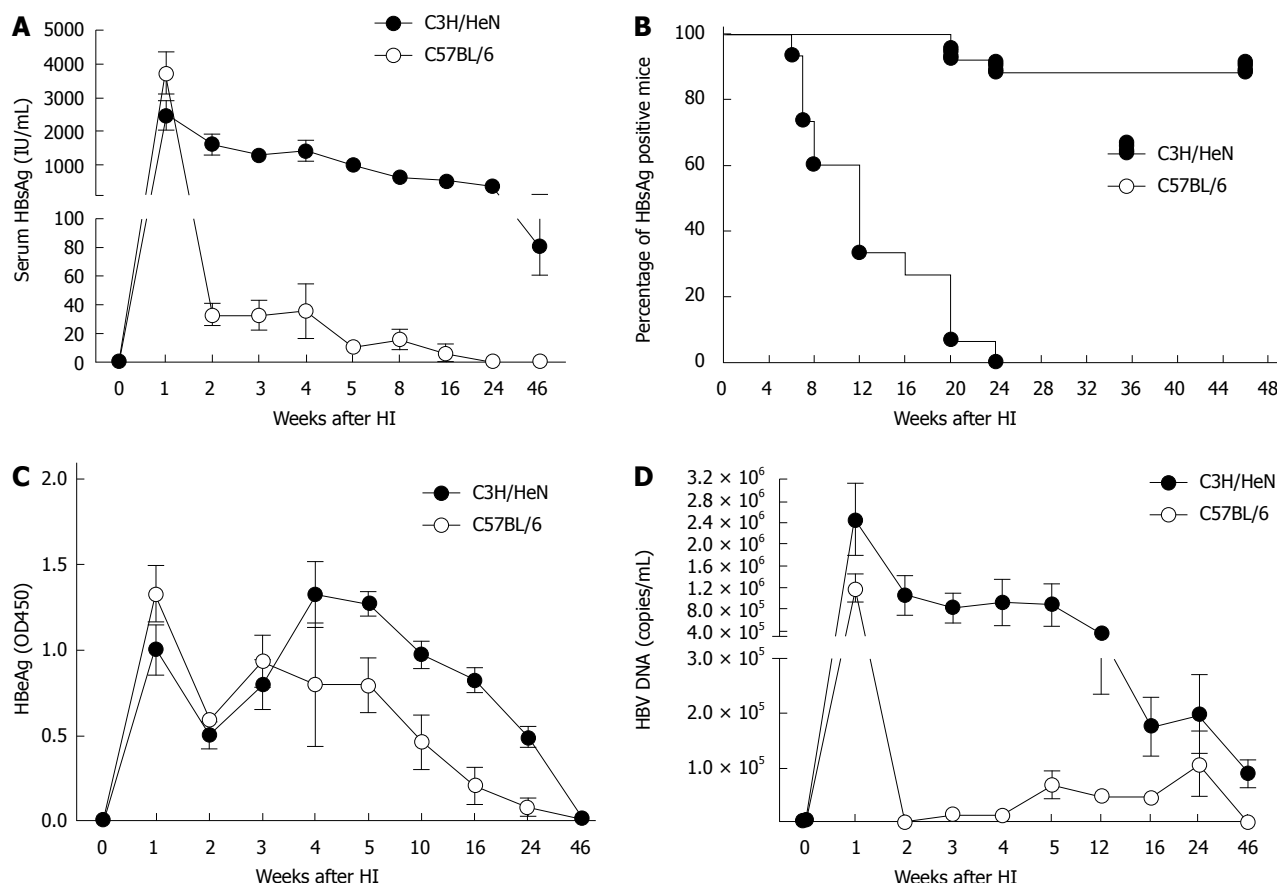


Figure 1 Hepatitis B surface antigen, hepatitis B e antigen and hepatitis B virus DNA levels in serum of C3H/HeN and C57BL/6 mice after hydrodynamic injection. pAAV/HBV1.2 DNA was injected hydrodynamically into the tail vein of 5-6 wk male C3H/HeN and C57BL/6 mice. After injection, the mice were regularly bled to monitor the serum levels of HBsAg, HBeAg and HBV DNA. A: Titer of serum HBsAg in C3H/HeN or C57BL/6 mice after HI at different time points; B: Positive rates of serum HBsAg in C3H/HeN ($n = 25$) or C57BL/6 ($n = 20$) mice at different time points after HI; C: Titer of serum HBeAg in C3H/HeN or C57BL/6 mice after HI at different time points; D: Serum HBV DNA level was determined at different time points. HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HI: Hydrodynamic injection.

differences were defined as $P \leq 0.05$.

RESULTS

HBV persists longer in hydrodynamically injected C3H/HeN mice than in C57BL/6 mice

In the hydrodynamically injected C57BL/6 mice, the HBsAg level increased promptly within 1 wk after pAAV/HBV1.2 injection but dropped quickly thereafter. In C3H/HeN mice, the HBsAg level declined much more slowly after injection of the same plasmid. Even at 46 wk post HI, it was still detectable (Figure 1A). Approximately 88% (22/25) of the injected C3H/HeN mice were still HBsAg-positive at 46 wk post HI whereas HBsAg in C57BL/6 mice was completely cleared at 24 wk (Figure 1B). Serum levels of HBeAg were increased to a peak quickly within a week, then decreased at 2 wk post HI, and increased again. Serum levels of HBeAg in C3H/HeN mice were higher than those in C57BL/6 mice from 4 wk to 46 wk (Figure 1C). Serum samples from hydrodynamically injected C3H/HeN and C57BL/6 mice were also assayed for

the presence of HBV DNA. In the hydrodynamically injected C3H/HeN mice, HBV DNA levels were higher than those in hydrodynamically injected C57BL/6 mice. At 46 wk post HI, HBV DNA in the C3H/HeN mice could still be detected, but it was undetectable in the C57BL/6 mice (Figure 1D).

Quantification of intrahepatic HBV DNA

We further examined the existing way of the liver HBV DNA. The liver tissues were collected from hydrodynamically injected C57BL/6 mice, C3H/HeN mice at 46 wk post injection, and naive mice as controls. The liver total DNA was assayed for the presence of encapsidated HBV DNA by real-time PCR. We used 0.5 μ L DpnI to digest the potential free plasmids and then also assayed the HBV DNA level by real-time PCR. Interestingly, we found that the liver HBV DNA level was not significantly changed between before and after DpnI digestion (Figure 2). We surmise that the DNA in serum HBsAg-positive C3H/HeN mice at 46 wk was not from free plasmid of pAAV/HBV1.2, but could be from actively replicating cytoplasmic

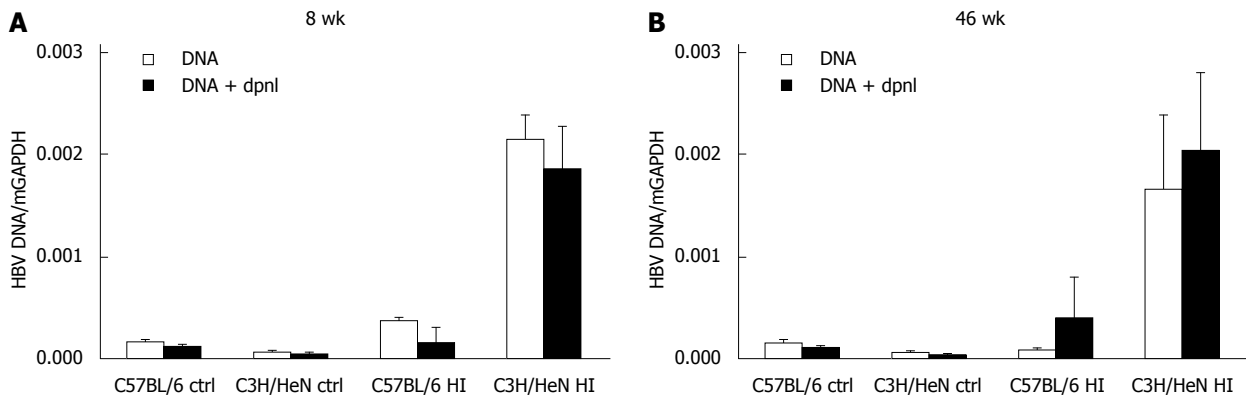


Figure 2 Quantification of intrahepatic hepatitis B virus DNA in the hydrodynamically injected mice. C3H/HeN or C57BL/6 mice were hydrodynamically injected with 10 μ g of pAAV/HBV1.2 plasmid, and the livers of hydrodynamically injected C3H/HeN ($n = 3$) or hydrodynamically injected C57BL/6 ($n = 3$) mice were collected at 8 wk or 46 wk after HI. Naive C3H/HeN ($n = 3$) or C57BL/6 ($n = 3$) mice were controls (ctrl). A: HBV DNA/mGAPDH in C3H/HeN or C57BL/6 mouse liver total DNA with or without 0.5 μ L DpnI digestion at 8 wk post HI; B: HBV DNA/mGAPDH in C3H/HeN or C57BL/6 mouse liver total DNA with or without 0.5 μ L DpnI digestion at 46 wk post HI. HBV: Hepatitis B virus; HI: Hydrodynamic injection.

nucleocapsid.

Hepatitis B core antigen and HBsAg expression in the liver of hydrodynamically injected mice

Liver tissues were collected from hydrodynamically injected C3H/HeN and C57BL/6 mice. Immunohistochemical staining was performed to determine the expression of hepatitis B core antigen (HBcAg) and HBsAg in the liver of the mice at 0, 5, 8, 46 wk post HI. As shown in Figure 3A, the number of HBcAg positive cells in C3H/HeN mice were not significantly different from that in C57BL/6 at 5 wk post HI. At 8 and 46 wk post HI, both cytoplasmic and nuclear HBcAg were detected in the liver of C3H/HeN mice but not in C57BL/6 mice. The HBsAg expression in the liver of C3H/HeN and C57BL/6 mice was not significantly different at 5 wk post HI, but was obviously higher in C3H/HeN than in C57BL/6 mice at 8 wk and 46 wk post HI, as shown in Figure 3B.

Impaired HBsAg-specific T cell immunity in C3H/HeN mice after HI

Specific T cell responses against HBV antigens (such as HBsAg) have been suggested to play critical roles in viral clearance^[12]. Here we addressed whether HBsAg-specific immunity is associated with the HBV persistence/clearance in C3H/HeN and C57BL/6 mice at 10 wk post HI of pAAV/HBV1.2 or untreated controls (Figure 4A). We examined the T cell responses against HBsAg two weeks after the final vaccination with HBsAg by IFN- γ -ELISPOT in hydrodynamically injected C3H/HeN mice, hydrodynamically injected C57BL/6 mice, control C3H/HeN and control C57BL/6 mice, and the average frequency was 0 , 17 ± 7 , 18 ± 10 , and 41 ± 10 SFCs/ 10^6 splenocytes, respectively. The frequency was significantly lower in hydrodynamically injected C3H/HeN mice than in control C3H/HeN and C57BL/6 mice (Figure 4B), and the mean spot sizes showed the same pattern

(Figure 4C). In contrast to hydrodynamically injected C57BL/6 capable of responding to HBsAg vaccination, the hydrodynamically injected C3H/HeN mice showed a totally tolerant phenotype to HBV with no responses to HBsAg vaccination; whereas naive control C3H/HeN mice responded to HBsAg vaccination, though it was lower than that in control C57BL/6 mice. Even just stimulated with PMA and ionomycin, frequency of IFN- γ positive T cells in the hydrodynamically injected C3H/HeN mice was much lower than that in control C57BL/6 mice ($P < 0.05$), and a little bit lower than those in hydrodynamically injected C57BL/6 and control C3H/HeN mice (Figure 4D). These data indicated that the HI of HBV genome into C3H/HeN mice could impair the T cell function in these mice, both specifically (*i.e.*, HBsAg-specific T-cell immunity) and globally.

IFN- α and entecavir treatment decreases HBV DNA in hydrodynamically injected C3H/HeN mice

Next, we tested whether this novel HI based C3H/HeN mouse model could be applied to the drug evaluation for HBV. IFN- α and entecavir treatment was performed in those hydrodynamically injected C3H/HeN mice. In the group of intragastric administration with entecavir (0.1 mg/kg, daily), real-time PCR analysis showed that serum HBV DNA levels declined (131.2-fold, $P < 0.01$) and kept going down 7 d post HI (Figure 5). In the group of IFN- α treatment (1 mg/kg, twice a week), serum HBV DNA levels declined to a lowest point (6.42 folds, $P < 0.05$) 7 d post HI and rebounded from then on (Figure 5). These data indicated that this HI based C3H/HeN mouse model can be used for antiviral drug evaluation for HBV.

DISCUSSION

Due to the narrow host restriction of HBV, the ideal experimental animal model of HBV should be

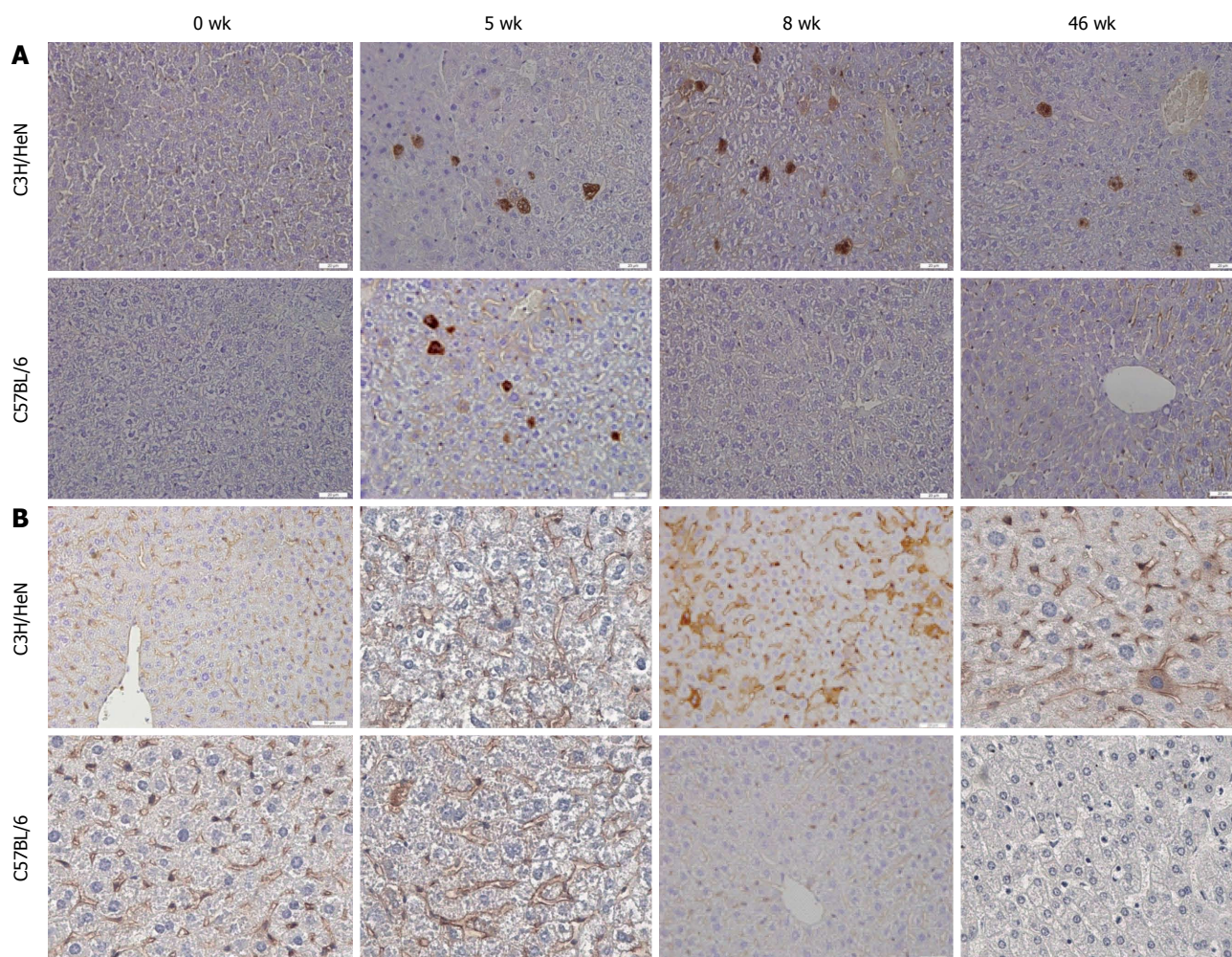


Figure 3 Longer expression of hepatitis B core antigen and hepatitis B surface antigen in the liver of C3H/HeN mice than C57BL/6 mice. C3H/HeN or C57BL/6 mice were hydrodynamically injected with 10 μ g of pAAV/HBV1.2 plasmid, and the livers of C3H/HeN or C57BL/6 mice were collected at 5, 8 and 46 wk after HI. A: Detection of HBcAg expression in C3H/HeN or C57BL/6 mouse liver at 5, 8 and 46 wk after HI by immunochemistry; B: Detection of HBsAg expression in C3H/HeN or C57BL/6 mouse liver at 5, 8 and 46 wk after HI by chemistry. Images of specific antibody stained slides were observed under a microscope at magnification of x 400. Experiments were repeated twice with a similar pattern. HBcAg: Hepatitis B core antigen; HBsAg: Hepatitis B surface antigen; HI: Hydrodynamic injection.

transgenic mice expressing human receptors for HBV. However, it was elusive for a long time about the receptors mediating HBV entry, though Li's group recently identified that NTCP (sodium taurocholate cotransporting polypeptide) was a functional receptor for human HBV^[13]. Whether there are coreceptors is still under investigation. Therefore, the success of HBV receptor-transgenic mice could be a long way to reach yet, considering the fact that the HCV receptors (CD81, SCARB1, CLDN1, OCLN) have been identified for a long time, but the HCV receptor-transgenic mouse has not succeeded until now^[14].

Current available animal models for HBV studies include duck HBV (DHBV)^[15] and woodchuck HBV (WHV)^[16] infection in their natural hosts, HBV-infected chimpanzees^[17], and HBV transgenic mice^[18]. DHBV and WHV are genetically different from HBV, and it is difficult to perform immunological studies in those animals due to their uncharacterized background. Chimpanzees are not available easily, and the high cost as well as ethical considerations limit their applications

to HBV study. HBV transgenic mice were widely used, but the drawback of immunological tolerance to the virus limits its applications in HBV immunological studies.

The HBV genome can be introduced into the mouse liver by transduction based on viral vectors, for example, adenovirus or adeno-associated viral vectors (AAVs) containing HBV DNA^[19,20]. Transduction of the HBV genome into mice using viral vectors leads to efficient viral gene expression in the liver and host immune response against HBV. However, the viral vector-induced immune responses (such as induction of type I IFN and other innate immune responses) may interfere with the host immune responses against HBV.

HI can efficiently deliver DNA into the liver *in vivo*. The DNA internalization by this hydraulic pressure-based physical transfection is receptor-independent and can achieve delivery to approximately 10%-40% of hepatocytes^[5,21]. Yang *et al.*^[22] first reported an acute HBV infection model by this method in B10.D2

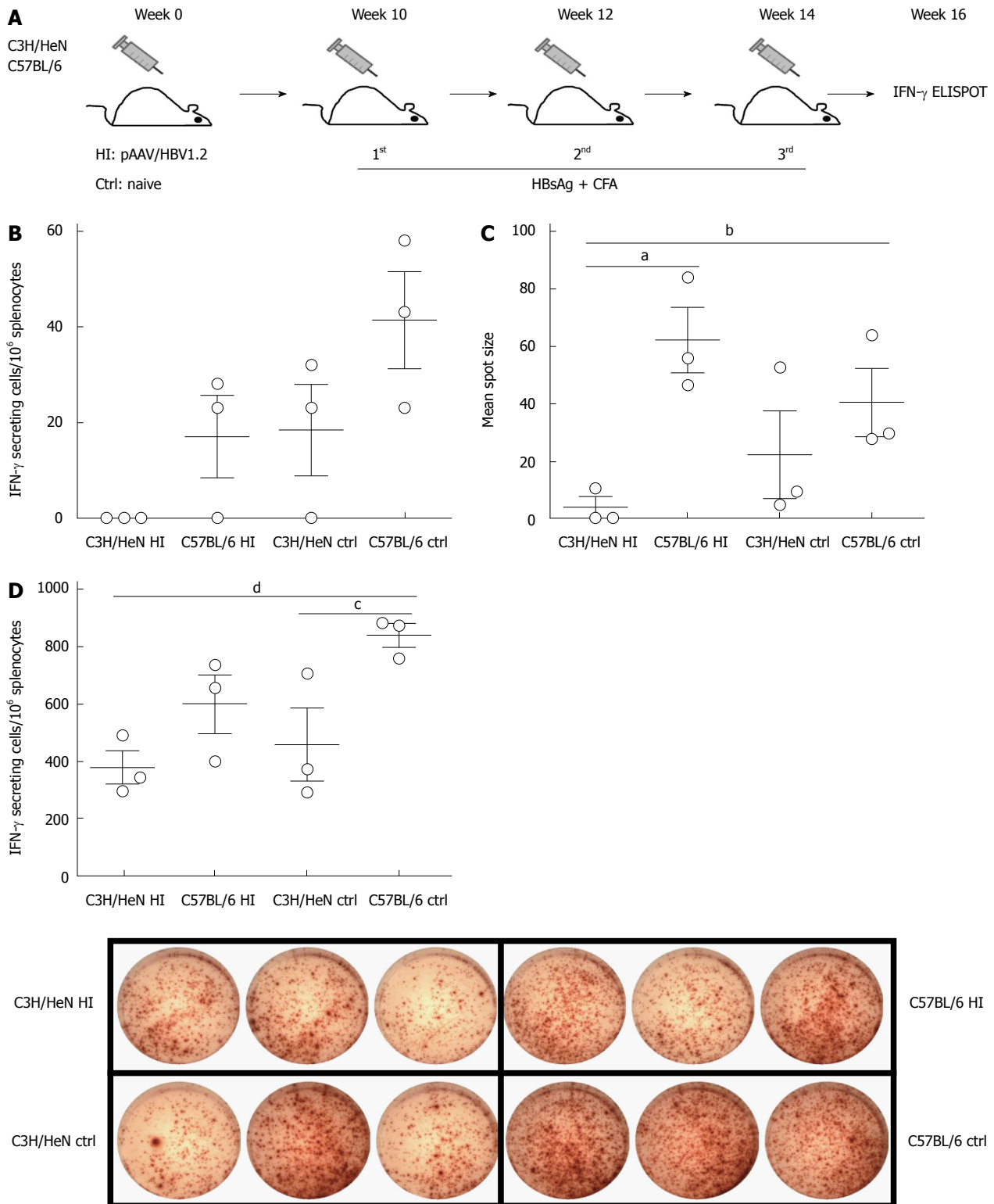


Figure 4 Hepatitis B surface antigen-specific T cell responses are impaired in hydrodynamically injected C3H/HeN mice. **A:** Regime of immunization: hydrodynamically injected C3H/HeN mice ($n = 3$) and C57BL/6 mice ($n = 3$) were subcutaneously injected with 5 μ g HBsAg protein formulated in CFA at 10, 12, and 14 wk after HI. Age-matched C3H/HeN naive mice ($n = 3$) and C57BL/6 naive mice ($n = 3$) were subcutaneously injected with 5 μ g HBsAg protein formulated in CFA three times at a 2-wk interval. Magnitudes of the total T cell responses were analyzed by IFN- γ ELISPOT two weeks after the final vaccination; **B:** Frequencies of IFN- γ producing T cells in C3H/HeN and C57BL/6 mice (both hydrodynamically injected ones and controls, $n = 3$ each group) after HBsAg stimulation. While hydrodynamically injected C3H/HeN mice showed almost zero in the frequency of IFN- γ positive cells, the other groups did show IFN- γ positive cells; **C:** Spot sizes of IFN- γ producing T cells in C3H/HeN and C57BL/6 mice (both hydrodynamically injected ones and controls, $n = 3$ each group) after HBsAg stimulation. Hydrodynamically injected C57BL/6 and control (ctrl) mice were significantly larger than hydrodynamically injected C3H/HeN mice [$^aP < 0.05$, C57BL/6 HI vs C3H/HeN HI mice; $^bP < 0.01$ control (ctrl) mice vs C3H/HeN HI mice, respectively]; **D:** IFN- γ producing T cell spot in C3H/HeN and C57BL/6 mice (both hydrodynamically injected ones and controls, $n = 3$ each group) after PMA + ionomycin stimulation; left: bar chart of frequencies of IFN- γ producing T cells. Hydrodynamically injected C57BL/6 mice were significantly higher than hydrodynamically injected C3H/HeN mice ($^cP < 0.01$, C57BL/6 HI vs C3H/HeN HI mice), and C3H/HeN mice ($^dP < 0.05$, C57BL/6 HI vs C3H/HeN); right: Images of ELISPOT. HI: Hydrodynamic injection; CFA: Complete Freund's adjuvant; IFN: Interferon; HBsAg: Hepatitis B surface antigen.

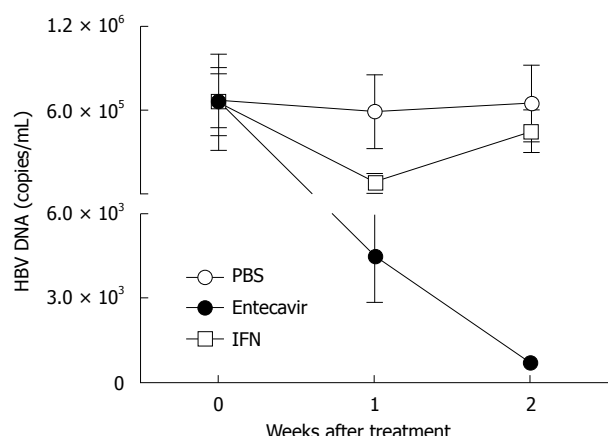


Figure 5 Interferon- α or entecavir treatment decreases hepatitis B virus DNA in hydrodynamically injected C3H/HeN mice. HI mouse model of HBV was created in 5-6-wk-old male C3H/HeN mice. IFN- α , entecavir or PBS treatment was performed on those mice 5 wk post HI. The black dot group ($n = 5$) was intragastrically administrated with 0.1 mg/kg entecavir once a day for 14 d; the open square group ($n = 5$) was intraperitoneally injected with 1 mg/kg IFN- α twice a week for 2 wk; the open dot group was treated with PBS as a control. Serum specimens were collected and assayed for HBV DNA on days 0, 7 and 14 after treatment. HBV: Hepatitis B virus; HI: Hydrodynamic injection; IFN: Interferon.

mice and persistent expression of HBV antigens was observed in hepatocytes of immunocompromised CB17 NOD/SCID mice. Huang *et al.*^[8] further reported that delivery of the HBV genome into immunocompetent mouse liver by HI could induce HBV hepatitis with very different rates of viral clearance. Both plasmid backbone and genetic background of recipient mice contributed to the long-term maintenance of HBV in the mouse liver. The plasmid pAAV/HBV1.2 is better than the plasmid pGEM4A/HBV1.2, though both of them harboured replication-competent HBV DNA^[8]. By using the same pAAV/HBV1.2, all BALB/c showed a rapid clearance of viral DNA template, whereas about 30% of the C57BL/6 mice showed the persistence of HBV^[8]. However, the bottleneck of a relative low rate of persistence of HBV in C57BL/6 mice compromised its potential strength in chronic HBV infection studies.

The different persistence rates of HBV in hydrodynamically injected BALB/c (H-2^d) and C57BL/6 (H-2^b) mice encourage efforts to find more suitable mouse strains for optimisation of the HBV persistent rate. Indeed, Chen *et al.*^[23] reported that long-term maintaining of HBV antigenemia can be detected in FVB/N (H-2^q) mice receiving HI of pGEM4Z/HBV1.3, and around 85% (6/7) of the mice were positive at 50 wk, compared with rapid clearance of HBV antigenemia in BALB/c mice within 4 wk and in C57BL/6 within 8 wk.

Our study successfully established HBV persistence in another inbred strain, C3H/HeN mice (H-2^k), through HI of pAAV/HBV1.2 plasmid. Around 90% (22/25) of the injected C3H/HeN mice were HBsAg-positive and the HBcAg positive cells in the liver were detected at 46 wk, though the detailed mechanisms are not clear yet. Chang *et al.*^[24] reported an acute HBV hepatitis model by hydrodynamically injecting pHBV3.6

into 8-12-wk-old C3H/HeN mice. The age of C3H/HeN mice might be critical for the different persistence rates between their model and ours. Actually, we found 5-6-wk-old C3H/HeN mice are best for persistence of HBV after HI, and the mice older than 8 wk showed a similar acute hepatitis to Chang Wang's model (data not shown).

It was well established that host immune responses contributed to HBV clearance^[5]. Chang *et al.*^[24] reported that C3H/HeJ mice, which had a defect in TLR4 signaling, showed higher HBV antigenemia and viral replication than C3H/HeN in the acute model (viraemia within 2 wk), indicating that TLR4 mediated innate immune response played a role in the HBV clearance. Adaptive immune responses, especially HBV-specific T cell response, are most critical for HBV clearance^[12]. Indeed, we found the HI of the HBV genome into C3H/HeN mice could impair the T cell function, both globally and specifically (*i.e.*, HBsAg-specific T-cell immunity), in contrast to hydrodynamically injected C57BL/6 mice capable of responding to HBsAg vaccination. These results were consistent with the previous reports on correlations between persistence of HBV in hydrodynamically injected mice and few activated specific cytotoxic T cells. Hence, C3H/HeN (H-2K) mice, which were like FVB/N (H-2q) mice^[23], were weaker in induction of HBV specific T cell responses than C57BL/6 (H-2b) mice; and the latter might be weaker than BALB/c (H-2d) mice^[8]. These findings suggested that HI of the HBV genome could cause tolerance to HBV in the hydrodynamically injected C3H/HeN mice and this tolerance might be largely related to the HBV persistence phenotypes in those mice.

Taken together, though the route of viral genome delivery by hydrodynamic-based transfection is different from that of natural infection *via* receptors, this immunocompetent non-transgenic mouse model can mimic the nature course of chronic HBV infection in human to a great extent. Our novel hydrodynamically injected C3H/HeN mice with a high persistence rate of HBV described in this study could provide a new approach to dissect the immunomechanism of HBV clearance or persistence, a new platform to evaluate the antiviral drugs against HBV, and a new model to analyse the different pathogenicities of clinical HBV isolates.

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COMMENTS

Background

The research of hepatitis B virus (HBV) infection, either studies on immunological mechanisms or therapeutic drug development, has been hampered by the shortage of suitable small animal models, albeit hydrodynamic injection (HI) of plasmids containing replication-competent HBV genome *via* the tail vein into immune-competent mice has been a model for HBV studies. However, in the case of classic HI model using C57BL/6 mice, only about 30% of the injected mice carried HBV for more than 12 wk, which limits its applications especially in the studies of chronic hepatitis B (CHB).

Research frontiers

In this manuscript the authors injected the pAAV-HBV1.2 plasmid into a different inbred mouse strain, C3H/HeN, and observed that the serum hepatitis B surface and hepatitis B e antigens and viral DNA persisted even up to 46 wk in about 90% of the injected mice, while almost all of the injected C57BL/6 mice, as controls, cleared HBV at 24 wk. The authors also detected HBsAg, HBeAg and HBV DNA expression in the liver tissues of the C3H/HeN mice at 46 wk post HI. Moreover, those mice showed impaired ability to induce HBsAg specific T cells responses, which is an important phenotype of immune tolerance to HBV. Applying IFN- α or entecavir (an analog of guanosine) in this HI model decreased HBV DNA *in vivo*.

Innovations and breakthroughs

The mouse background can affect the result of the long-term maintenance of HBV in the mouse liver. After HI of the same pAAV/HBV1.2, BALB/c mice showed a rapid clearance of viral DNA template, whereas about 30% of the C57BL/6 mice showed the persistence of HBV (Huang *et al.*). However, the bottleneck of a relative low rate of the persistence of HBV in the C57BL/6 mice compromised its potential strength in chronic HBV infection studies. Thus, efforts were encouraged to find more suitable mouse strains for optimisation of the HBV persistent rate. Indeed, Chen *et al.* reported that a long term maintaining of HBV antigenemia can be detected in FVB/N (H-2^b) mice receiving HI of pGEM4Z/HBV1.3, and around 85% (6 in 7) of the mice were positive at 50 wk, compared with rapid clearance of HBV antigenemia in BALB/c mice within 4 wk and in C57BL/6 within 8 wk. This study successfully established HBV persistence in another inbred strain, C3H/HeN mice (H-2^k), through HI of pAAV/HBV1.2 plasmid. Around 90% of the injected C3H/HeN mice were HBsAg-positive and the hepatitis B core antigen positive cells in the liver were detected at 46 wk. The data came from an observation in 25 mice/group and could be more solid. In addition, more inbred strains (including H-2^k background) available for HI HBV models could expand the possibilities to study the genetic factors on HBV persistence. Chang *et al.* reported an acute HBV hepatitis model by hydrodynamically injecting pHBV3.6 into 8-12-wk-old C3H/HeN mice. The age of C3H/HeN mice might be critical for the different persistence rates between their model and ours. Actually, this study showed 5-6-wk-old C3H/HeN mice are best for persistence of HBV after HI.

Applications

The results in this paper suggested that the HI-based HBV infection model using the C3H/HeN mice might provide a more stable platform for mechanistic research of CHB and therapeutic development.

Terminology

HI means injection with 10 μ g endotoxin-free plasmid DNA into the tail vein of mouse, in a volume of PBS equivalent to 10% of the mouse body weight, and the total volume was delivered within 5 s. HI can efficiently deliver DNA into the liver *in vivo*. The DNA internalization by this hydraulic pressure-based physical transfection is receptor-independent and can achieve delivery to approximately 10%-40% of hepatocytes. This method has been used to establish HBV transfection mouse models, classically in C57BL/6 mice.

Peer-review

The paper showed that HBV persisted longer in C3H/HeN (H-2k) mice after the HI compared with C57BL/6 (H-2b) mice, suggesting that host genetic background determines the rate of HBV clearance. The authors suggested that this could be a novel animal model for CHB infection to elucidate the disease pathogenesis and develop new antiviral treatments. Overall, the study showed a clear association between mouse genetic background and the rate of persistence. However, the authors should elucidate the mechanistic basis for the frequent persistence in the C3H/HeN (H-2k) mice.

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Basic Study

Therapeutic effect of Qingyi decoction in severe acute pancreatitis-induced intestinal barrier injury

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the expression of secreted phospholipase A₂ (sPLA₂) in intestinal barrier injury.

METHODS: Fifty healthy Sprague-Dawley rats were randomly divided into control, severe acute pancreatitis (SAP), Qingyi decoction-treated (QYT), dexamethasone-treated (DEX), and verapamil-treated (VER) groups. The SAP model was induced by retrograde infusion of 1.5% sodium deoxycholate into the biliopancreatic duct of the rats. All rats were sacrificed 24 h post-SAP induction. Arterial blood, intestine, and pancreas from each rat were harvested for investigations. The levels of serum amylase (AMY) and diamine oxidase (DAO) were determined using biochemical methods, and serum tumor necrosis factor (TNF)- α level was measured by an enzyme linked immunosorbent assay. Pathologic changes in the harvested tissues were investigated by microscopic examination of hematoxylin and eosin-stained tissue sections. The expressions of sPLA₂ at mRNA and protein levels were detected by reverse transcriptase PCR and Western blot, respectively. A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay was used to investigate apoptosis of epithelial cells in the intestinal tissues.

RESULTS: Compared to the control group, the expression of sPLA₂ at both the mRNA and protein levels increased significantly in the SAP group (0.36 ± 0.13 vs 0.90 ± 0.38 , and 0.16 ± 0.05 vs 0.64 ± 0.05 , respectively; $P_s < 0.01$). The levels of AMY, TNF- α and DAO in serum were also significantly increased (917 ± 62 U/L vs 6870 ± 810 U/L, 59.7 ± 14.3 ng/L vs 180.5 ± 20.1 ng/L, and 10.37 ± 2.44 U/L vs 37.89 ± 5.86 U/L, respectively; $P_s < 0.01$). The apoptosis index of intestinal epithelial cells also differed significantly between the SAP and control rats (0.05 ± 0.02 vs 0.26 ± 0.06 ; $P < 0.01$). The serum levels of DAO and TNF- α , and the intestinal apoptosis index significantly correlated with sPLA₂ expression in the intestine ($r = 0.895$, 0.893 and 0.926 , respectively; $P_s < 0.05$). The

Abstract

AIM: To investigate the effect of Qingyi decoction on

levels of sPLA₂, AMY, TNF- α , and DAO in the QYT, VER, and DEX groups were all decreased compared with the SAP group, but not the control group. Qingyi decoction intervention, however, gave the most therapeutic effect against intestinal barrier damage, although the onset of its therapeutic effect was slower.

CONCLUSION: Qingyi decoction ameliorates acute pancreatitis-induced intestinal barrier injury by inhibiting the overexpression of intestinal sPLA₂. This mechanism may be similar to that of verapamil.

Key words: Intestinal barrier injury; Qingyi decoction; Secreted phospholipase A₂; Severe acute pancreatitis; Verapamil

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Core tip: Secreted phospholipase A₂ (sPLA₂) is a damage factor that stimulates excessive inflammatory responses, which can lead to the degradation and hydrolysis of biologic membranes, thus promoting epithelial injury. We demonstrate that sPLA₂ is overexpressed at the mRNA and protein levels in a rat model of severe acute pancreatitis-induced intestinal barrier injury. However, a traditional Chinese medicine, Qingyi decoction, effectively antagonized this overexpression of sPLA₂ to alleviate the severity of the disease. This observation was comparable to the inhibitory effect of verapamil on sPLA₂ expression.

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INTRODUCTION

Severe acute pancreatitis (SAP) is a common surgical acute abdominal disease and can lead to the early death of patients because of associated systemic inflammatory response syndrome and multiple organ dysfunction syndrome^[1]. One severe complication is often intestinal barrier damage. This permits great quantities of gut bacteria and endotoxin to enter the blood and lymphatic circulations, and eventually whole internal organs^[2]. Also, the body's mononuclear macrophage system is activated, which leads to release of large quantities of tumor necrosis factor (TNF), interferons, interleukins, and other inflammatory factors to trigger cascade of inflammatory events that further cause tissue damage^[3,4].

Secreted phospholipase A₂ (sPLA₂), the main phospholipase A subtype, is a damage factor whose function

depends on intracellular calcium ion concentration. Under the stimulus of excessive sPLA₂ expression, large amounts of inflammatory factors cause the degradation of cell surface active substances and hydrolyze biologic membranes to aggravate lecithin damage in organs and tissues^[5,6]. The efficacy of the Chinese medicine Qingyi decoction (QYT) has been demonstrated through clinical practice and animal experiments for years; QYT is an effective prescription for the treatment of acute pancreatitis^[6]. It is generally well tolerated by patients, and induces purgation, promotes blood circulation, eliminates blood stasis, and reduces inflammation. It can also directly neutralize endotoxins and protect the intestinal barrier. In our previous study on the intervening role of QYT in patients following acute pancreatitis, it was shown that QYT administration reduced lung injury by decreasing the transcription of sPLA₂, thereby protecting pulmonary function^[6].

Dexamethasone (DEX) is a glucocorticoid with several beneficial functions including anti-inflammatory activity, microcirculation promotion, and oxygen free radical scavenging. Verapamil (VER) can effectively reduce tissue damage, especially intestinal damage, by reducing intracellular calcium ion concentration which plays a decisive role in sPLA₂ activation^[7]. VER is a commonly used calcium blocker. Considering its inhibitory effect on sPLA₂, VER may be used to protect against intestinal tissue and pancreatic injuries during SAP.

The present study aimed to examine the role of sPLA₂ in a rat model of SAP-induced intestinal barrier injury and the intervening roles of QYT and VER.

MATERIALS AND METHODS

Animals and grouping

Fifty clean-grade healthy male Sprague-Dawley rats (180-220 g, age: 8 wk) were purchased from the specific-pathogen-free Animal Center of Dalian Medical University (Dalian, China). The animals were randomly divided into five groups ($n = 10$ per group): controls, untreated SAP, and SAP treated with QYT (Chinese Medicine Preparations Division, First Affiliated Hospital of Dalian Medical University, Dalian, China, Supplementary 1), DEX (Ling Rui Pharmaceutical, Zhengzhou, China), or VER (Harvest Pharmaceutical, Shanghai, China). This study was carried out in strict accordance with the recommendations in the European Union Animal Management Practices (1986). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Dalian Medical University (Dalian, China).

Model preparation

The SAP intestinal barrier damage model was established using bile pancreatic duct retrograde injection of 1.5%

deoxycholic acid sodium salt (Baier Di Biotechnology, Beijing, China). The rats were subject to preoperative fasting of 12 h with free access to drinking water, and administered 10% chloral hydrate anesthesia (*ip*, 3 mL/kg) prior to operation. Under sterile conditions, the needle of a 1 mL syringe was inserted into the major duodenal papilla of the rat and 1.5% deoxycholic acid sodium salt (1 mL/kg dose, speed of 0.1 mL/min) was injected through the bile pancreatic duct into the pancreas. The control group only had their pancreas marginally rotated to avoid any incidence of mild acute pancreatitis that could arise following the injection of the solvent (water) used to dissolve the salt. The very short time (24 h) required for the manifestation of chemically induced SAP would not permit complete resolution of such mild acute pancreatitis in the control rats, which would in turn compromise the principal clinical differences between the control and the SAP groups. The DEX group (10 mg/kg body weight/dose, 5 mg/mL concentration) and VER group (1.25 mg/kg body weight/dose, 2.5 mg/mL concentration) were given their respective drugs intravenously immediately, 6 and 12 h post-operation. The QYT group, however, was orally treated with QYT (10 mL/kg body weight/dose) 0.5 h before the induction of SAP (to permit enough time for the absorption of the traditional drug into the blood), and then 6 and 12 h post-operation. At 24 h post-operation, animals were anesthetized and abdominal aortic blood was taken for serum collection and storage at -80 °C until use. The pancreas and intestinal tissues were harvested, and part of each was either immediately stored at -80 °C or fixed in neutral phosphate formaldehyde.

Pathologic observation

Pathologic observations were made under an optical microscope (Leica DMIRB; Leica, Solms, Germany). Pancreas and intestinal tissues previously fixed in neutral phosphate formaldehyde were paraffin embedded and sectioned (2 µm serial sectioning) for pathologic morphology observation after routine hematoxylin and eosin staining.

Serum amylase, TNF-α, and diamine oxidase measurement

Serum amylase content was determined using a fully automatic biochemical analyzer (Abbott Laboratories, ML, United States) from 50 µL of rat serum that was diluted six times with MilliQ (Millipore Corp, Billerica, MA, United States) water before acquisition.

Serum TNF-α level was determined using an enzyme-linked immunosorbent assay kit (Lengton Company, Shanghai, China) according to the manufacturer's instructions.

For diamine oxidase (DAO) detection, 80 µL of serum was added to 800 µL of detection reagent (Tris-HCl, reduced coenzyme, glutamate dehydrogenase, 1.4 d diamine mixture), mixed, and incubated for 20 s, and

absorbance was read at 340 nm wavelength for the value of A1. The mixture was placed in a water bath (37 °C) for 10 min, and then the absorbance at 340 nm was read again for the A2 value. The DAO activity (U/L) = $\{[A1 \times A2]/[A2 \times \text{cuvette diameter (cm)} \times 6.3 \times \text{NADH mmol extinction coefficient}] \times [\text{reaction liquid volume (µL)}/\text{sample volume (µL)}]\} \times 1000$.

Detection of intestinal epithelial cell apoptosis

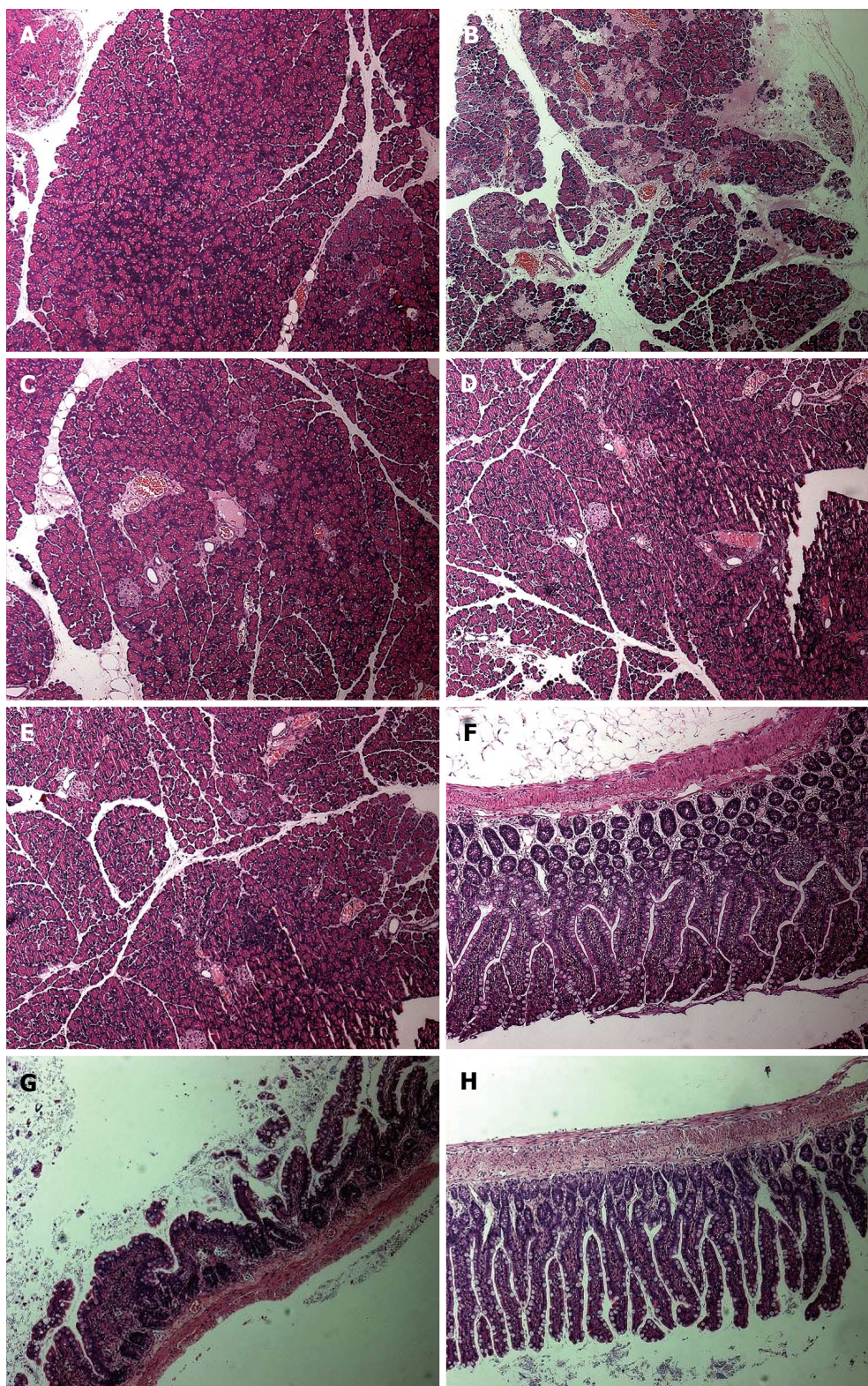
A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay kit (Keygen, Nanjing, China) was used to detect apoptosis of epithelial cells in paraffin-embedded intestinal tissues according to the manufacturer's instructions. Under high magnification (200 ×), five randomly selected areas were observed. The apoptosis index (AI) was calculated as the percentage of fluorescein isothiocyanate-positive cells out of 500 intestinal epithelial cells.

Quantitative PCR assessment of intestinal sPLA₂ mRNA expression

Total RNA was extracted from intestinal tissue using RNAisoPlus (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. Briefly, 1 mL of RNAisoPlus was added to 50 mg of tissue and homogenized on ice. Then 200 µL of chloroform was added and the mixture centrifuged before the supernatant was collected. Isopropyl alcohol was used to precipitate the RNA from the chloroform. Precipitated RNA was dissolved in RNase-free water and its quality measured using an ultraviolet spectrophotometer. The observed values of A260/A280 ranged between 1.8 and 2.0. The routine PCR condition included 30 cycles at an annealing temperature of 60 °C for 30 s. Primers (Takara) were as follows: sPLA₂, 5'-GTGGCAGGATCCCCCAAGG-3' (upstream), 5'-GCAACTGGGCGTGTTCCTCTGCA-3' (downstream), product length, 283 bp; and β-actin, 5'-GGAGTCCTGTGGCATCCACG-3' (upstream), 5'-CTAGAAGCATTGCGGTGGA-3' (downstream), product length, 531 bp. An ultraviolet imaging system (Protein Simple; Alphalmade HP, Santa Clara, CA, United States) was used to read the PCR products after agarose gel electrophoresis. Intestinal tissue sPLA₂ mRNA expression level was estimated as the ratio of intestinal sPLA₂ mRNA gray value to its corresponding internal control (β-actin) gray value.

Western blotting for intestinal tissue sPLA₂ protein expression

Total protein was extracted using 1 mL of RIPA lysis buffer supplemented with 1 µL protease inhibitor solution, 5 µL PMSF and 10 µL phosphatase inhibitor (all purchased from Keygen, Nanjing, China) for every 100 mg of intestinal tissue; protein (100 µg) from each sample was separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Sections of the membrane were cut according to the estimated



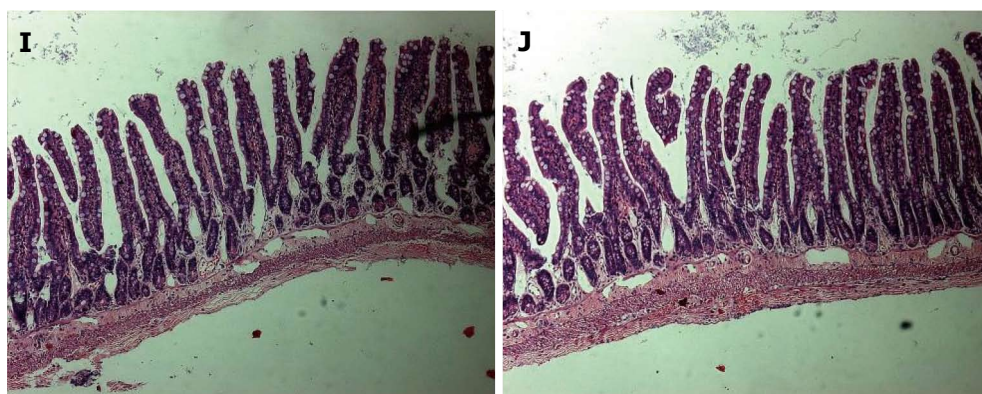


Figure 1 Pathologic changes in the pancreatic and intestinal tissues of the experimental model groups. Pancreatic tissue in A: Control; B: Severe acute pancreatitis (SAP); C: SAP treated with Qingyi decoction (QYT); D: SAP treated with dexamethasone (DEX); and E: SAP treated with verapamil (VER) rats. Intestinal tissue in F: Control; G: SAP; H: QYT-treated; I: DEX-treated; and J: VER-treated rats (hematoxylin and eosin, 100 ×).

Table 1 Serum amylase, tumor necrosis factor- α , and diamine oxidase levels, and apoptosis index of the different experimental groups

Group (<i>n</i> = 12)	AMY (U/L)	TNF- α (ng/L)	DAO (U/L)	AI
Control	917 \pm 62	59.7 \pm 14.3	10.37 \pm 2.44	0.05 \pm 0.02
SAP	6870 \pm 810 ^b	180.5 \pm 20.1 ^b	37.89 \pm 5.86 ^b	0.26 \pm 0.06 ^b
QYT	4048 \pm 511 ^a	122.4 \pm 15.2 ^a	22.43 \pm 2.13 ^a	0.13 \pm 0.04 ^a
DEX	3363 \pm 200 ^a	137.0 \pm 23.4 ^a	24.27 \pm 3.36 ^a	0.16 \pm 0.03 ^a
VER	3852 \pm 234 ^a	125.2 \pm 16.5 ^a	26.96 \pm 5.56 ^a	0.19 \pm 0.03 ^a

AMY: Serum amylase; TNF: Tumor necrosis factor; DAO: Diamine oxidase; AI: Apoptosis index; SAP: Severe acute pancreatitis; QYT: Qingyi decoction; DEX: Dexamethasone; VER: Verapamil; ^a*P* < 0.05 *vs* SAP; ^b*P* < 0.01 *vs* controls.

molecular weight of proteins of interest and blocked in 5% non-fat milk at 37 °C for 1 h, then incubated in blocking buffer with diluted primary antibody (sPLA₂, 1:400 or β -actin, 1:1000; Santa Cruz Biotechnologies, Dallas, TX, United States) at 4 °C overnight with gentle rocking. After incubation, the membrane was rinsed three times and incubated in HRP-conjugated secondary antibody (1:10000) at 37 °C with gentle rocking for 1 h, then rinsed three times and bands visualized using enhanced chemiluminescence followed by film exposure. Band intensities were analyzed using ImageJ software, version 1.35d (National Institutes of Health, Bethesda, MD, United States).

Statistical analysis

Statistical analysis was conducted using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, United States). All data are reported as mean \pm SD, and analysis of variance was used for comparisons among the groups. A *P* < 0.05 was considered statistically significant. The Pearson product-moment correlation was used for correlation analysis.

RESULTS

Pancreatic and intestinal tissue histopathology

Substantial pathologic changes were observed in the

pancreatic and intestinal tissues from each group of rats except the control group, where mucosa lobular structures did not exhibit edema or bleeding. Other features observed in the disease groups included blurred SAP pancreas lobular structures, large numbers of inflammatory cell infiltration, extensive hemorrhaging, and intestinal epithelial cell necrosis of > 50%. However, the pathologic damage in QYT, DEX, and VER groups was greatly reduced compared to the SAP group. The QYT and VER groups exhibited intestinal mucosal epithelial cell swelling, generally of normal morphology, whereas the DEX group, in addition to cell swelling deformation, showed varying degrees of inflammatory cell infiltration. Their pancreatic lobule structures were clear with only a small amount of edema, hemorrhage, and inflammatory cell invasion. Necrotic areas were the smallest compared to the DEX and SAP groups (Figure 1).

Serum levels

Compared with the control group, serum AMY levels in the SAP rats significantly increased (*P* < 0.01) (Table 1). However, serum AMY levels in the treated (QYT, DEX or VER) groups were significantly lower compared with the SAP group (*P*s < 0.05). Furthermore, serum TNF- α levels were significantly elevated in the SAP group compared to controls (*P* < 0.01). The serum TNF- α levels significantly decreased in the SAP rats following QYT, DEX, or VER intervention (*P*s < 0.05). Serum DAO levels in the SAP group were significantly higher than controls (*P* < 0.01). Compared with the SAP group, levels of serum DAO in the QYT, DEX and VER groups decreased significantly (*P*s < 0.05).

Intestinal epithelial cell apoptosis

The intestinal epithelial cell AI in the SAP group was significantly higher compared with controls (*P* < 0.01). However, compared with the SAP group, the AIs of the QYT, DEX, or VER groups were significantly lower (*P*s < 0.05). The AI of the QYT group was the most reduced among the treated cohort (Table 1) (Figure 2).

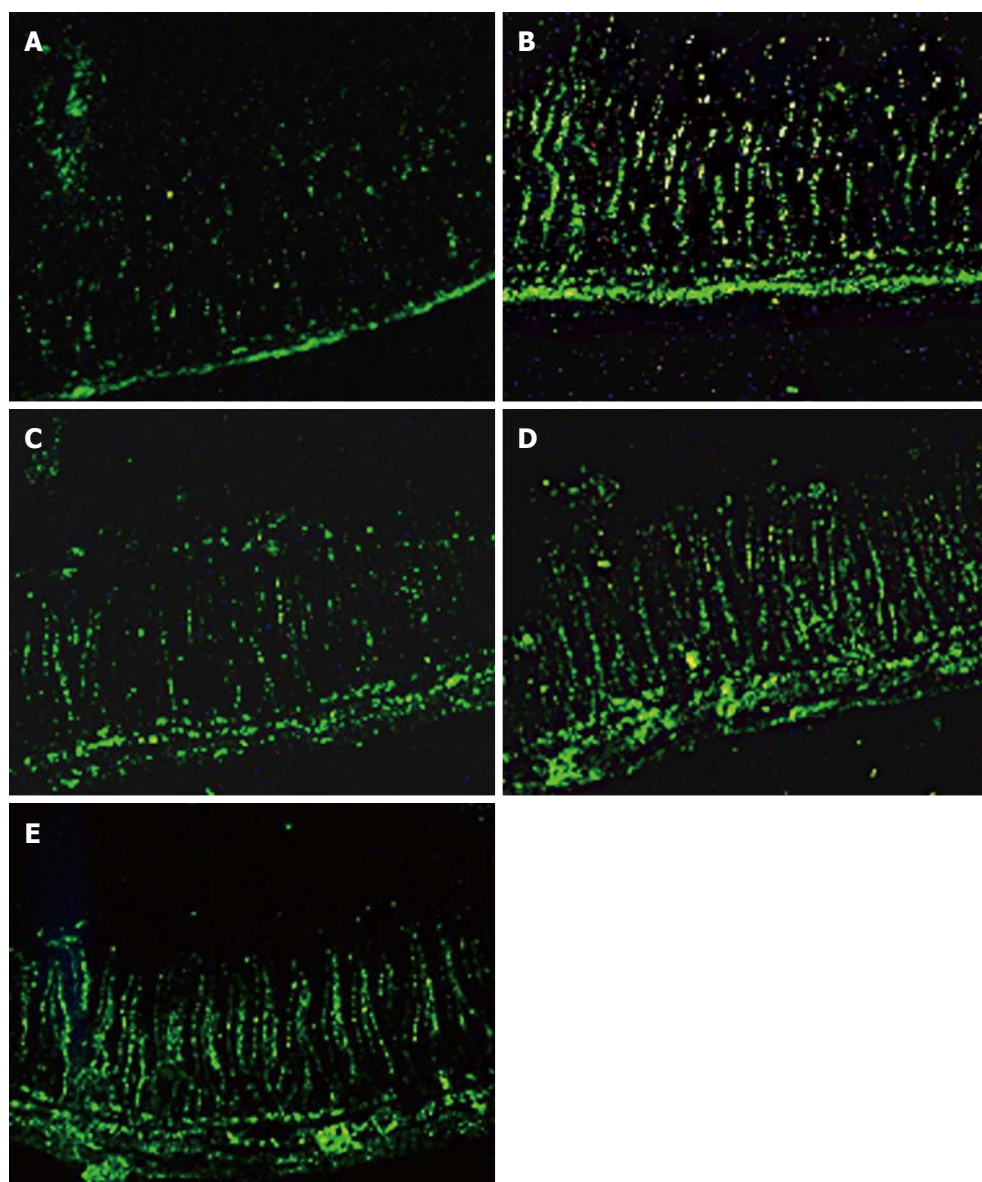


Figure 2 Intestinal epithelial cell apoptosis of the experimental model groups. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay in A: Control; B: Severe acute pancreatitis (SAP); C: SAP treated with Qingyi decoction; D: SAP treated with dexamethasone; E: SAP treated with verapamil rats (200 ×).

sPLA₂ mRNA expression in intestinal tissue

Compared with the control group, intestinal tissue sPLA₂ mRNA expression in the SAP group was significantly higher ($P < 0.01$) (Figure 3). The sPLA₂ mRNA expression level in the QYT, DEX, and VER groups, however, were significantly lower compared with the SAP group ($P_s < 0.05$).

sPLA₂ protein expression in intestinal tissue and correlation analysis

The expression of sPLA₂ protein in the intestinal tissue of the SAP group was significantly higher compared with the control group ($P < 0.01$) (Figure 4). Upon intervention with QYT, DEX, or VER in SAP rats, the protein level decreased significantly ($P_s < 0.05$). The inhibition of the sPLA₂ protein expression was superior in the QYT treatment group. Nonetheless, the intestinal

expression of sPLA₂ protein in the QYT, DEX, and VER groups was significantly higher compared with controls ($P_s < 0.05$). The protein expression level of sPLA₂ positively correlated with serum TNF- α and DAO levels in the SAP rats ($P_s < 0.05$) (Table 2).

DISCUSSION

sPLA₂ is widely present in mammalian tissues and cells, and functions to rebuild phospholipids, transmit signals in cell physiologic processes, and plays an important role in some diseases, such as SAP^[7]. Overexpression of sPLA₂ promotes a large release of arachidonic acid, prostaglandin, platelet-activating factors, and other bioactive substances^[8-10]. Overexpression of sPLA₂ is mainly stimulated by a large number of inflammatory mediators, and it is an important factor in intestinal

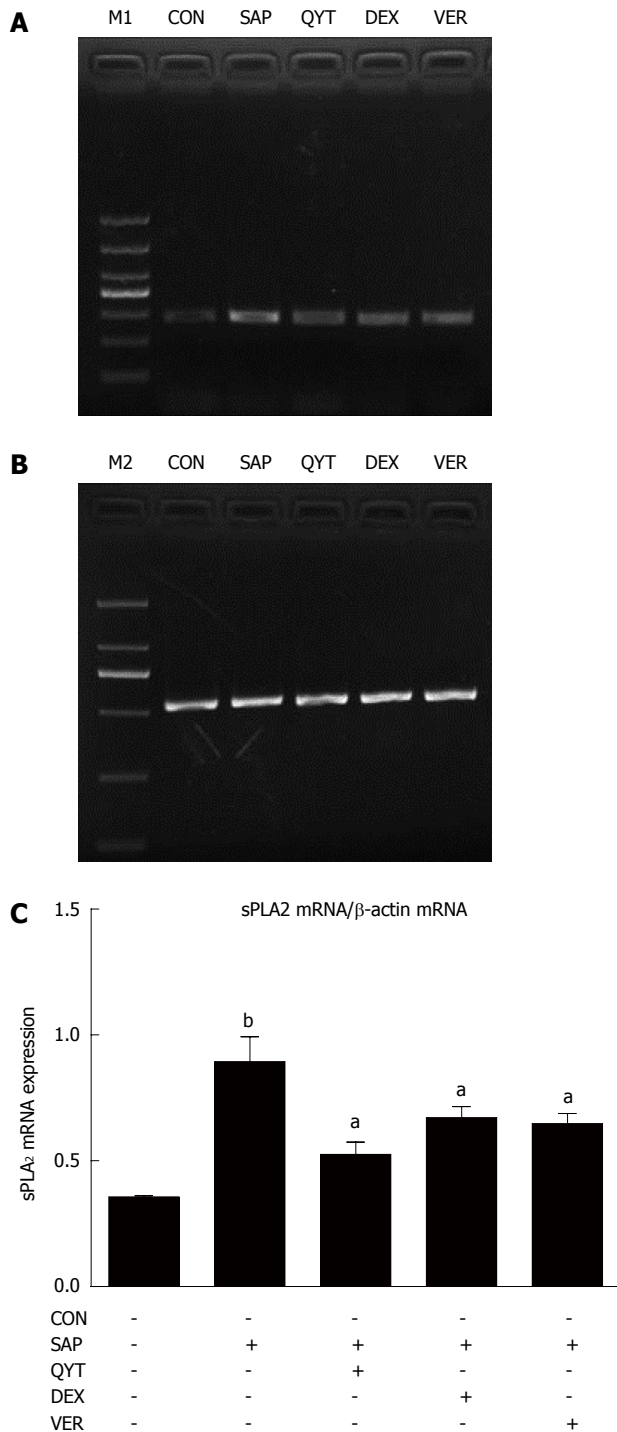


Figure 3 Electrophoresis micrographs of secreted phospholipase A₂ and β-actin mRNA quantitative PCR products. Electrophoresis micrograph of A: sPLA₂ mRNA (283 bp); and B: β-actin (as internal control; 531 bp); C: Gray value ratio of sPLA₂ to β-actin mRNA expression. ^a*P* < 0.05 vs SAP; ^b*P* < 0.01 vs CON. CON: Control; DEX: Dexamethasone; M1: 1000 bp-marker; M2: 2000 bp-marker; QYT: Qingyi decoction; SAP: Severe acute pancreatitis; VER: Verapamil.

ischemia-reperfusion injury^[11,12]. sPLA₂ can degrade phospholipid components of cell membranes, and thus directly damage the intestinal mucosa. It can also indirectly cause ischemia-reperfusion injury and deregulate the inflammatory cytokine network, and

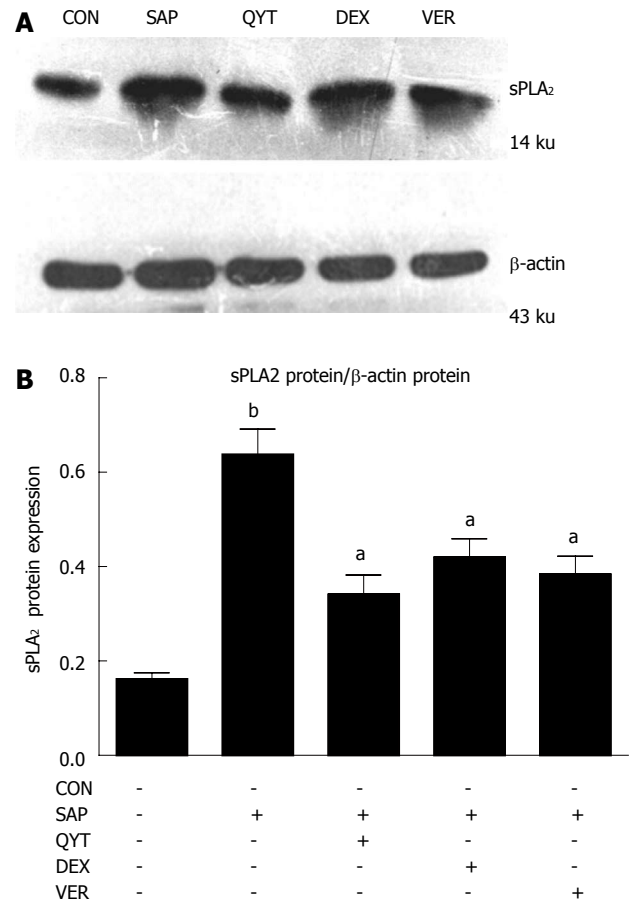


Figure 4 Secreted phospholipase A₂ protein expression in intestinal tissue. A: Western blotting for intestinal tissue sPLA₂ protein expression (β-actin was used as the loading control); B: Gray value ratio of sPLA₂ to β-actin protein expression. ^a*P* < 0.05 vs SAP; ^b*P* < 0.01 vs CON. CON: Control; DEX: Dexamethasone; QYT: Qingyi decoction; SAP: Severe acute pancreatitis; VER: Verapamil.

thus attack the intestinal barrier in the development and progression of SAP^[13-15]. The overall consequences include decreased intestinal peristalsis and intestinal epithelial cell apoptosis and necrosis^[16-18]. These activities may promote the multiplication of intestinal bacteria and their translocation into the blood, which can cause gut origin sepsis and endotoxemia^[19-21]. Destruction of the intestinal mucosa mechanical barrier also results in the release of a large amount of diamine oxidase enzymes and active substances^[22-24]. Wilmore *et al.*^[25] first proposed that intestinal barrier injury may lead to systemic inflammatory response syndrome and multiple organ dysfunction syndrome^[26-30]. Therefore, the study of the role of sPLA₂ in the pathogenesis of intestinal barrier damage in SAP is of great significance.

We found that SAP successfully developed in the rats after 24 h of disease induction, characterized by pancreatic and intestinal tissue injury, and pathologic changes. The serum levels of AMY, TNF-α, and DAO significantly increased in the SAP group compared with either the controls or any of the treated groups.

Table 2 Correlation analysis of secreted phospholipase A₂ with serum diamine oxidase and tumor necrosis factor- α levels, and apoptosis index

Statistic	DAO	TNF- α	AI
<i>r</i>	0.895	0.893	0.926
<i>P</i> -value	< 0.05	< 0.05	< 0.05

TNF: Tumor necrosis factor; DAO: Diamine oxidase; AI: Apoptosis index.

Intestinal epithelial cell apoptosis index and sPLA₂ expression at both the mRNA and protein levels were also significantly increased in the SAP group. Additionally, the expression level of sPLA₂ positively and significantly correlated with serum TNF- α and DAO levels. The extent of damage to the intestinal barrier positively correlated with intestinal sPLA expression level, thus suggesting the involvement of sPLA₂ in the process of intestinal barrier damage in SAP.

QYT, DEX and VER demonstrated the potential to reduce damage to the pancreatic and intestinal tissues. These drugs also decreased serum AMY, TNF- α , and DAO levels, the intestinal epithelial cell AI, and the expression of sPLA₂ mRNA and protein. However, the interventional effect of QYT and VER were better. Thus, administration of QYT may help reduce symptoms of intestinal paralysis^[31]. QYT is also suggested to promote the inhibition of intestinal phospholipase overexpression, the release of inflammatory mediators and toxic substances, and reduce the proliferation of intestinal bacteria and the effect of their endotoxin, which when in the blood, triggers systemic inflammation^[9,26]. However the precise mechanism by which QYT protects intestinal damage is yet to be comprehensively elucidated.

DEX is commonly used clinically as an anti-inflammatory agent as it inhibits inflammation and inflammation promoters, decreases vascular permeability, and antagonizes phospholipase A₂-induced release of platelet-activating factor to ease inflammation and reduce tissue injury^[32-34]. This study used DEX as a reference drug to compare the therapeutic effects of QYT and VER on intestinal barrier damage during SAP progression in rats. Both experimental drugs effectively reduced the expression of sPLA₂ at the transcriptional and translational levels as compared to the reference drug, and offered superior therapeutic effect against the extent of intestinal barrier damage. Preliminary results (data not shown) indicated that sPLA₂ expression was significantly higher in intestinal mucosa than in the lung tissue of SAP rats. Thus, suggesting that the role of sPLA₂ in intestinal barrier injury deserves attention.

Six hours post-operation and drug administration, the vitality of the rats in a descending order was in the DEX, QYT, and VER groups. However, 24 h post-operation, vitality was superior and disease progression was also gentler in the QYT and VER groups compared with the DEX group. The onset of

QYT therapeutic effect, although slower, persisted longer compared with DEX. QYT has been widely used in clinical settings, particularly for the treatment of SAP and lung injury^[35,36]. Acute pancreatitis is currently diagnosed based on clinical staging, disease evolution, and other characteristics. Its treatment may be greatly enhanced if Western orthodox medicine is combined with traditional medicinal preparations, such as QYT, for a synergistic therapeutic effect, and also to ameliorate some of the commonly associated complications, including intestinal barrier injury.

COMMENTS

Background

Severe acute pancreatitis (SAP) can lead to the early death of patients because of associated systemic inflammatory response syndrome and multiple organ dysfunction syndromes. However, its pathogenesis has not been fully elucidated, which has impaired the development and availability of specific clinical treatments to date.

Research frontiers

It is currently recognized that intestinal barrier injury is the initiating factor for SAP-associated multiple organ failure.

Innovations and breakthroughs

The therapeutic function of the traditional Chinese medicine, Qingyi decoction, was comparable to the Western orthodox drug verapamil in a rat model of SAP. Qingyi decoction, although slower in onset of action, effectively inhibited the overexpression of secreted phospholipase A₂ (sPLA₂), which is known to play an essential pathologic role in the development of intestinal barrier injury, a common complication in SAP.

Applications

The intestinal transcription and protein expression levels of sPLA₂ positively correlated with the serum levels of proinflammatory factors tumor necrosis factor- α and diamine oxidase, and therefore, may be of diagnostic and/or prognostic significance in SAP disease.

Peer-review

SAP remains a serious clinical problem with significant morbidity and mortality. Studies suggest that loss of the gut barrier function is instrumental in the local and systemic infectious complications associated with a severe course of the disease. Improvement of intestinal barrier function may be a useful strategy to alleviate the severity and possibility of infectious complication in SAP. This study explored the involvement of sPLA₂ in intestinal barrier injury in SAP, and the intervening role of Qingyi decoction and verapamil in comparison with dexamethasone as a reference treatment. Qingyi decoction is a traditional Chinese prescription in treatment of SAP. This study is interesting and important to the field.

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Case Control Study

Surgical outcomes of Korean ulcerative colitis patients with and without colitis-associated cancer

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Ethics approval: The study was reviewed and approved by the Institutional Review Board of Asan Medical Center (IRB No. 2013-0683).

Informed consent: Because this was a retrospective study with minimal risk to patients, it was exempted from obtaining informed consent by the IRB.

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Abstract

AIM: To determine the clinicopathologic characteristics of surgically treated ulcerative colitis (UC) patients, and to compare the characteristics of UC patients with colitis-associated cancer (CAC) to those without CAC.

METHODS: Clinical data on UC patients who underwent abdominal surgery from 1980 to 2013 were collected from 11 medical institutions. Data were analyzed to compare the clinical features of patients with CAC and those of patients without CAC.

RESULTS: Among 415 UC patients, 383 (92.2%) underwent total proctocolectomy, and of these, 342 (89%) were subjected to ileal pouch-anal anastomosis. CAC was found in 47 patients (11.3%). Adenocarcinoma was found in 45 patients, and the others had either neuroendocrine carcinoma or lymphoma. Comparing the UC patients with and without CAC, the UC patients with CAC were characteristically older at the time of diagnosis, had longer disease duration, underwent frequent laparoscopic surgery, and were infrequently given preoperative steroid therapy ($P < 0.001-0.035$). During the 37 mo mean follow-up period, the 3-year

overall survival rate was 82.2%.

CONCLUSION: Most Korean UC patients experience early disease exacerbation or complications. Approximately 10% of UC patients had CAC, and UC patients with CAC had a later diagnosis, a longer disease duration, and less steroid treatment than UC patients without CAC.

Key words: Ulcerative colitis; Colorectal neoplasms; Colorectal surgery; Survival; Inflammatory bowel disease

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Core tip: This multi-center study is the first nationwide report on the surgical outcomes of Korean ulcerative colitis (UC) patients and reflects the recent status of surgically treated Korean UC patients. The authors found that most Korean UC patients experienced early disease exacerbation or complications. Approximately 10% of UC patients had colitis-associated cancer (CAC), and UC patients with CAC had a later diagnosis, a longer disease duration, and less steroid treatment than UC patients without CAC.

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INTRODUCTION

Despite the growing use of medical salvage treatment, surgery, including total proctocolectomy (TPC), remains a cornerstone for managing ulcerative colitis (UC). Surgery should be regarded as a life-saving procedure for patients with acute severe colitis and must be seriously considered in any medically intractable patient or patient with colonic dysplasia or malignancy^[1]. A recent study of ileal pouch-anal anastomosis (IPAA) showed excellent quality of life and a good functional outcome in UC patients treated with this modality^[2]. Colitis-associated cancer (CAC) is a well-recognized complication of UC^[3]. The overall prevalence of CAC in UC patients is 3.7%, and cases of CAC in UC patients account for only 1% of all colorectal cancer (CRC) cases observed in the Western population^[3,4]. There is also a general consensus that patients with longstanding, extensive UC have an increased risk of developing CAC^[3,5].

Although the prevalence of UC is lower in South Korea than in Western countries, the number of

patients with UC as well as those with UC and CAC has increased steadily since 1980^[6,7]. There are clear ethnic differences in inflammatory bowel disease (IBD) between Asian and Western populations^[8]. The present study is primarily intended to fulfill the current lack of information on the clinicopathologic characteristics of UC patients who undergo surgical treatment in South Korea. We also attempted to compare the clinical characteristics of surgically treated UC patients with and without CAC.

MATERIALS AND METHODS

Data collection

The data for biopsy-proven UC patients who underwent abdominal surgery from January 1980 to July 2013 were collected retrospectively. The surgeries were performed at 11 different medical institutions, *i.e.*, ten university hospitals and one colorectal clinic. The data of 419 patients were initially collected, and four patients were excluded because they had not undergone surgery for UC. Thus, data from a total of 415 UC patients were analyzed to compare the clinical variables of UC patients with cancer to those of patients without cancer. The variables were gender, family history, age at diagnosis, symptom duration before diagnosis, preoperative medication, the indication for surgery, the presence of primary sclerosing cholangitis (PSC), the extent of colonic involvement, the type of surgery, the postoperative complications, and mortality. In the patients with cancer, additional data were gathered, including preoperative identification of cancer or dysplasia, preoperative serum carcinoembryonic antigen (CEA) level, pathologic data, adjuvant chemotherapy or radiation therapy, recurrence of cancer, and survival status at the time of the last follow-up. Histologically, tumors were classified as either low-grade (well-differentiated or moderately differentiated adenocarcinoma) or high-grade (poorly differentiated adenocarcinoma or mucinous or signet-ring cell carcinoma). An early complication was defined as one occurring within 90 d after the main surgical intervention. The study protocol was approved by the Institutional Review Board of each medical institution. The mean follow-up was 68.4 mo (range: 0-286 mo).

Statistical analysis

A cross-table analysis using Pearson's χ^2 test or Fisher's exact test, as appropriate, was used to compare the discrete variables of patients with cancer to those of patients without cancer. The Student's *t*-test was used for between-group comparisons of continuous variables. Among UC patients with cancer, recurrence and overall survival were used to evaluate the clinical outcome. Survival outcomes were compared using the Kaplan-Meier method with a log-rank test. All reported *P* values are two-sided, and the *P* < 0.05 values were considered to indicate statistical significance. SPSS

Table 1 Patients' clinical characteristics according to the presence/absence of cancer *n* (%)

Variable	Total (<i>n</i> = 415)	UC w/o cancer (<i>n</i> = 368)	UC w/ cancer (<i>n</i> = 47)	<i>P</i> value
Gender, male	212 (51.1)	186 (50.5)	26 (55.3)	0.642
Age at diagnosis (yr)	38.4 ± 0.7	37.9 ± 0.7	42.7 ± 2.6	0.035
Age at surgery (yr)	43.4 ± 0.7	42.2 ± 0.8	52.9 ± 2.1	< 0.001
Period between diagnosis and surgery (yr)	5.0 ± 0.3	4.3 ± 0.3	10.2 ± 1.3	< 0.001
Symptomatic period (yr)	6.0 ± 0.3	5.3 ± 0.3	11.9 ± 1.2	< 0.001
Family history	5 (1.4)	3 (0.9)	2 (4.8)	0.103
Extracolonic manifestation	49 (11.8)	45 (12.2)	4 (8.5)	0.632
PSC	5 (1.2)	4 (1.1)	1 (2.1)	0.453
Preoperative steroid therapy	315 (75.9)	297 (80.7)	18 (38.3)	< 0.001
Location, pancolitis	320 (77.1)	284 (77.2)	36 (76.6)	0.025
Left side colitis	88 (21.2)	80 (21.7)	8 (17.0)	
Proctitis only	7 (1.7)	4 (1.1)	3 (6.4)	
Laparoscopic surgery	46 (11.1)	34 (9.2)	12 (25.5)	0.002
Overall complications	144 (34.7)	133 (36.1)	11 (23.4)	0.103
Early	79 (19.0)	75 (20.4)	4 (8.5)	0.050
Late	65 (15.7)	58 (15.8)	7 (14.9)	0.878
Mortality	5 (1.2)	5 (1.4)	0 (0)	0.925

UC: Ulcerative colitis; PSC: Primary sclerosing cholangitis.

Table 2 Surgical indications *n* (%)

Variable	Value
Medical intractability	270 (65.1)
Dysplasia or malignancy	52 (12.5)
Bleeding	31 (7.5)
Perforation	19 (4.6)
Toxic megacolon	18 (4.3)
Obstruction	9 (2.2)
Fistula	3 (0.7)
Others	13 (3.1)
Total	415 (100)

Table 3 Surgical procedures *n* (%)

Variable	Value
Total proctocolectomy	383 (92.2)
w/ IPAA	342 (89)
One-stage procedure	38 (9.2)
Two-stage procedure	286 (68.9)
Three-stage procedure	18 (4.3)
Mucosal proctectomy	53 (12.8)
w/ end ileostomy	41 (11)
Total colectomy w/ end ileostomy	11 (2.7)
Other colon surgery	21 (5.1)
Total	415 (100)

software version 18.0 (SPSS, Chicago, IL, United States) was used for statistical analysis.

RESULTS

Clinical characteristics of the patients

Table 1 shows the clinical characteristics of 415 UC patients who underwent surgical treatment. The mean preoperative medication period was 41.9 mo. Most of the patients (*n* = 368, 88.7%) were treated with 5-aminosalicylic acid (5-ASA). Steroids were administered to 315 patients (75.9%) as a first-line treatment for acute severe colitis before colectomy. In patients who failed to respond to steroids, infliximab (*n* = 33), cyclosporine (*n* = 26), and 6-mercaptopurine (*n* = 7) were used as second-line treatments. The most common reason for performing surgery was medical intractability, followed by dysplasia or malignancy, and bleeding (Table 2). With regard to surgical treatment, 383 patients (92.2%) underwent TPC, of which 342 patients (89%, Table 3) underwent IPAA. Among the 342 patients who underwent IPAA, 53 mucosectomies with hand-sewn IPAA (15.4%) were performed.

IPAA: Ileal pouch-anal anastomosis.

Mucosectomy was more frequently performed in patients with cancer (27.2%) than in those without cancer (14.7%), although the difference was not significant. Laparoscopic-assisted procedures were performed in 46 patients (11.1%). Complications occurred in 144 patients (34.7%), 79 of whom had early complications and 65 of whom had late complications. The most common early complication was ileus (*n* = 21), followed by bleeding (*n* = 16), anastomotic leakage (*n* = 15), intra-abdominal abscess (*n* = 8), and major wound dehiscence (*n* = 6). Late complications were pouchitis (*n* = 48), fistula (*n* = 9), and anastomotic stricture (*n* = 6). Preoperative steroid therapy was more frequently used in open surgery than in laparoscopic surgery (79% vs 50%, *P* < 0.001). The complication rate in patients undergoing preoperative steroid therapy was higher than that in patients who did not undergo preoperative steroid therapy (38% vs 26%, *P* = 0.04). There was no significant difference in the complication rates between open and laparoscopic surgery.

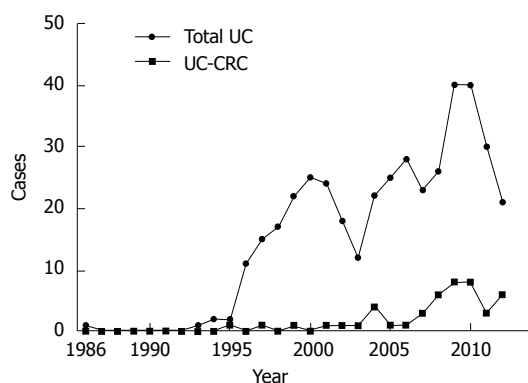


Figure 1 Yearly surgical cases of ulcerative colitis and ulcerative colitis-associated colorectal cancer in South Korea. Since 2006, the occurrence of ulcerative colitis-associated colorectal cancer (UC-CRC) has steadily increased.

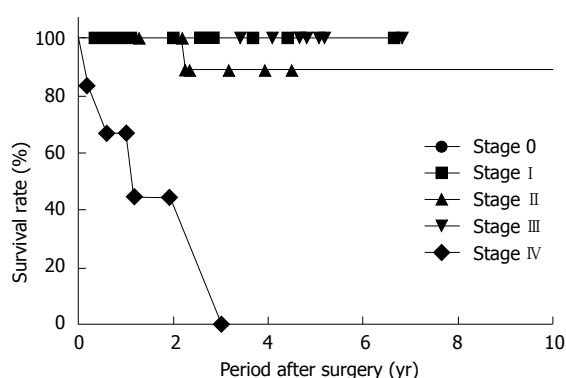


Figure 2 Survival curves according to the pathologic stage in ulcerative colitis patients with colorectal adenocarcinoma.

CAC in patients with UC

Forty-seven patients had colorectal malignancies (11.3%): 45 patients had adenocarcinomas, and the two had a neuroendocrine carcinoma and a lymphoma, respectively. There was a suspicion of malignancy before surgery in 44 patients (93.6%). Although there was no difference in the annual cumulative number of surgeries performed for UC, surgery for cancer with UC has been increasing recently (Figure 1). Compared with the UC patients without cancer, the UC patients with cancer were older at the time of diagnosis and surgery, and had longer disease duration, frequent laparoscopic surgery, infrequent preoperative steroid therapy, and a slightly lower rate of early postoperative complications (Table 1). Two patients were diagnosed with rectal adenocarcinomas 7 and 11 years after total colectomy with end ileostomy and underwent completion proctectomy. None of the patients had malignancy around the pouch or anal transitional zone (ATZ) after IPAA.

Table 4 summarizes the characteristics of the 45 UC patients with colorectal adenocarcinoma. Two patients with rectal cancer underwent preoperative chemoradiation therapy. Adjuvant chemotherapy was administered to 24 patients (53.3%) with advanced

Table 4 Characteristics of ulcerative colitis-associated colorectal adenocarcinoma *n* (%)

Variable	Value
Location	
Right colon	10 (22.2)
Left colon	18 (40.0)
Rectum	17 (37.8)
CEA	
< 6 ng/mL	33 (73.3)
≥ 6 ng/mL	6 (13.3)
Unknown	6 (13.3)
Tumor size (cm), mean ± SE	3.9 ± 0.4
< 4 cm	24 (53.3)
≥ 4 cm	15 (33.3)
Unknown	6 (13.3)
Histology	
Well-differentiated	10 (22.2)
Moderately differentiated	26 (57.8)
Poorly differentiated	4 (8.9)
Mucinous	5 (11.1)
Stage	
0	3 (6.7)
I	13 (28.9)
II	11 (24.4)
III	11 (24.4)
IV	7 (15.6)
Curability of the surgery	
R0	35 (77.8)
R1	3 (6.7)
R2	7 (15.5)
Total	45 (100)

CAC. During the 37-mo mean follow-up period (range: 1-138 mo), the 3-year overall survival rate was 82.2%. The only patient with local recurrence had stage II CAC. She was diagnosed with recurrent pelvic lymph-node metastasis 1 year postoperatively and finally died due to cancer progression. Among the seven patients with stage IV CAC, five died, and two patients were alive although with disease, at the end of the study period. In patients with stage 0, I, and III CAC, no recurrences or deaths were observed (Figure 2).

DISCUSSION

This multi-center study is the first nationwide report on the surgical outcomes of Korean UC patients and reports the recent status of surgically treated Korean UC patients. As our study involved most high-volume, tertiary-care, medical institutions in South Korea, we calculated that the patient cohort (approximately *n* = 5800 patients) at 11 of these institutions corresponded to approximately 45% of all recorded Korean patients with UC. This was indirectly calculated by counting the number of follow-up UC patients at each institution and using the population data of a previous KASID study^[4]. Recently, colorectal surgeons have reported encountering increasing numbers of UC patients with CAC in their clinical practice. Therefore, our study was designed primarily to determine the incidence

and characteristics of Korean UC patients with CAC. Unexpectedly, the number of these patients was relatively small, and their follow-up periods were too short to analyze survival outcomes.

In the clinical course of UC, approximately 4% to 9% of UC patients will require colectomy within the first year of diagnosis, whereas the risk of requiring colectomy after the first year is 1% per year^[9]. A European population-based study revealed a 7.5% colectomy rate during the 5-year follow-up period^[10]. In our study, two-thirds of the UC patients underwent surgery due to exacerbation of their disease (severe colitis), and approximately 20% of the UC patients underwent surgery due to complicating disease including massive bleeding or perforation. These patients had an average 4.3-year interval between diagnosis and surgery. Conversely, only 10% of the UC patients who underwent surgery had CAC. These patients had different clinical characteristics, such as a later diagnosis, disease duration of more than 10 years, and a lower rate of preoperative steroid therapy than patients without CAC.

Needless to say, the standard-of-care surgery for UC is TPC with IPAA, which most of the patients in this study received. IPAA is a curative and well-tolerated procedure, although it is technically demanding and has a high morbidity rate. A recent study of IPAA demonstrated early complications in 33%, and late complications in 29% of patients, thus resulting in an overall pouch excision rate of 5%^[2]. Our study showed slightly lower complication rates than those seen in Fazio's study. However, this is not surprising, considering that their database was prospectively well-maintained at a single medical institution and that our databases were collected at 11 medical institutions over a short period of time. IPAA can be performed in one, two, or three stages. In our study, 84% of the IPAAs were performed using a two-stage procedure, which gave similar results to those reported in a recent, large-scale cohort study^[2]. In patients undergoing a three-stage procedure, completion proctectomy or rectal surveillance is very important. A recent study reported that only 65% of patients completed IPAA after subtotal colectomy, 40% complied with rectal surveillance, and two patients developed rectal cancers, which is consistent with our study results^[11].

The "double stapled" ileal J pouch-anal anastomosis is the most popular standard pouch-anal anastomosis method, while mucosectomy and hand-sewn anastomosis are reserved for patients with dysplasia or cancer^[2,12]. However, whether mucosectomy protects against the development of ATZ and pouch cancer is unclear, and controversy exists over whether the beneficial effect of mucosectomy in preventing neoplasia is outweighed by its negative effect on ileal pouch function^[13]. Although mucosectomy was frequently performed on our UC patients with cancer, it was difficult to verify the benefits of mucosectomy

for preventing ATZ cancer due to the short follow-up period in our study. A recent review also showed that 32 UC patients had cancers in the ATZ; of these patients, 28 underwent mucosectomy. The study concluded that mucosectomy does not necessarily eliminate cancer risk in the ATZ^[14].

Laparoscopic IPAA for UC is feasible; however, to date, the evidence in the literature is still inconclusive. Current data suggest that it allows a shorter hospital stay, a shorter ileus, faster recovery, and less postoperative pain, along with better cosmesis when minimally invasive surgery is employed. Significantly longer operative times are universally reported when laparoscopy is employed^[15]. In our study, only 11% of the UC patients underwent laparoscopic-assisted surgery. Among these, the complication rates did not differ from those of open surgery and were closely correlated with the infrequent use of preoperative steroid therapy. Many studies suggested that patients who are taking high-dose steroids are at an increased risk of early complications after IPAA^[16,17]. As cumulative evidence shows that laparoscopic surgery for CRC is not inferior to open surgery, with respect to patient survival and cancer recurrence rates^[18], laparoscopic IPAA surgery might be feasible in selected UC patients with cancer.

Long disease duration, male sex, a young age at diagnosis of UC, extensive colitis, and PSC are well-known risk factors for developing CAC^[5,14,19]. The disease duration is the most important factor for UC-associated CRC, of which the incidence rates correspond to cumulative probabilities of 2% by 10 years, 8% by 20 years, and 18% by 30 years^[3]. As our study included UC patients who underwent surgery, it was difficult to determine the risk factors for UC-associated CRC. We also found that the duration of UC was longer in patients with CAC than in patients without CAC. A previous KASID study of UC-associated CRC in South Korea revealed that the overall prevalence of CRC was 0.37%, the mean age at diagnosis was 49.6 years, and the mean duration of UC was 11.5 years, all of which are consistent with our study results^[4]. During the KASID study period of 1970 to 2005, Kim *et al.*^[4] found 26 UC patients with CAC. However, 80% of the UC patients with CAC in our study were identified after 2005 and had earlier disease stages than those in the KASID study. Although it was difficult to identify changes in the management and preventive strategies for CAC during the study period, our findings might be explained by the increase in the UC cohort, as well as by the recent increase in the use of surveillance colonoscopy for the prevention of CAC. In addition, a very low incidence of PSC was consistently found in both studies. By comparing our results with those of the previous KASID study, we also verified that the incidence of UC-associated CRC is rapidly increasing.

Compared with sporadic CRC, the carcinogenesis of UC-associated CRC is different, as it develops from

dysplasia in a carcinogenic pathway known as the dysplasia-carcinoma sequence^[14]. Interestingly, *P53* mutations occur earlier in IBD-associated cancer than in sporadic CRC. *APC* mutations in IBD-associated cancer, a key initiating event, occur later than sporadic CRC^[20]. Furthermore, microsatellite instability (MSI) is frequently observed in UC patients^[21], although MSI in IBD shows infrequent MLH1 hyper-methylation, which is a dominant feature of sporadic CRC^[22]. These molecular genetic differences between IBD-associated cancer and sporadic CRC might be responsible for their different clinicopathologic features. The pathologic features of UC-associated CRC frequently present as a mucinous or signet-ring-cell histology compared with the features of sporadic CRC (17%-21%)^[23,24], which is consistent with our results (20%). A previous Japanese study indicated that a frequent mucinous or a signet-ring-cell histology in UC-associated cancer contributed to the poorer prognosis of UC-associated cancer compared with that of sporadic CRC^[23].

Whether the survival rate of UC-associated CRC is poorer than that of sporadic CRC is controversial. Earlier studies showed similar survival rates for UC-associated CRC and sporadic CRC^[25,26]. However, recent well-designed Danish and Japanese studies revealed slightly poorer survival rates for UC-associated CRC patients^[23,27]. In Norwegian and Swedish population-based studies, the prognosis of IBD-associated CRC was poorer than that of sporadic CRC (a mortality rate ratio of 3.71 for Norwegians and an overall hazard ratio of 1.26 for Swedes)^[28,29]. As all of these studies had the common limitation of a small patient cohort, it is difficult to obtain an accurate prognosis for UC-associated CRC patients. Although our study also had the same limitation of a small number of cases of UC-associated CRC as well as a short follow-up period, the survival of UC patients with CAC in our study was much better than that seen in recent studies. Except for stage IV patients, among 38 patients with stage 0 to III disease, only one with a recurrence died. Long-term follow-up and further patient enrollment might help provide an accurate prognosis for Korean UC patients with CAC in the future.

This study had some of the limitations of a retrospective study. There were differences in the reliabilities of the databases, which differed from institution to institution. Although a few institutions had prospectively well-maintained databases, others did not. As we previously mentioned, our study population was very limited as it only included patients who underwent surgery. Therefore, it is difficult to determine the risk factors for UC-associated CRC from this cohort.

In conclusion, Korean UC patients who underwent surgery had two distinct features. Most of the treated patients had early disease exacerbation or complications. Approximately 10% of the surgically treated UC patients had CAC and characteristics of late diagnosis, longer disease duration, and lower

preoperative steroid treatment compared with those without CAC.

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COMMENTS

Background

Despite the advancements in medical treatment, surgery remains a cornerstone for managing ulcerative colitis (UC). Surgery should be regarded as a life-saving procedure for patients with acute severe colitis and patients with colonic malignancy. Colorectal cancer is a well-recognized complication of long-term UC.

Research frontiers

Although the prevalence of UC is low in Asian countries, the number of patients with UC as well as those with colorectal cancer has increased steadily since 1980. There are clear ethnic differences in inflammatory bowel disease between Asians and Westerners. The present study addressed the current lack of information concerning the surgical treatments and outcomes of UC patients by determining the clinicopathologic characteristics of Korean UC patients who underwent surgical treatment.

Innovations and breakthroughs

Only 10% of surgically treated UC patients had colitis-associated cancer. These patients were characteristically older at the time of diagnosis, had longer disease duration, underwent frequent laparoscopic surgery, and were infrequently given preoperative steroid therapy. Surgical outcomes of UC-associated cancer patients were similar to those of sporadic colorectal cancer patients.

Applications

Understanding the clinicopathologic characteristics of UC-associated colorectal cancer might help better manage UC patients and make optimal decisions about which type of surgery to apply.

Peer-review

This is an interesting multicenter study of the clinic-pathologic characteristics of patients with UC who underwent surgery.

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Case Control Study

Primary biliary cirrhosis-associated hepatocellular carcinoma in Chinese patients: Incidence and risk factors

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Abstract

AIM: To investigate the incidence, characteristics, and risk factors for hepatocellular carcinoma (HCC) in Chinese patients with primary biliary cirrhosis (PBC).

METHODS: We reviewed the data of 52 PBC-associated HCC patients treated at Beijing 302 Hospital from January 2002 to December 2013 and analyzed its incidence and characteristics between the two genders. The risk factors for PBC-associated HCC were analyzed *via* a case-control study comprising 20 PBC patients with HCC and 77 matched controls without HCC. The matched factors included gender, age, follow-up period and Child-Pugh scores. Conditional logistic regression was used to evaluate the odds ratios of potential risk factors for HCC development. A $P < 0.05$ was considered statistically significant.

RESULTS: The incidence of HCC in Chinese PBC patients was 4.13% (52/1255) and was significantly higher in the males (9.52%) than in the females (3.31%). Among the 52 PBC patients with HCC, 55.76% (29/52) were diagnosed with HCC and PBC simultaneously, and 5.76% (3/52) were diagnosed with HCC before PBC. The males with PBC-associated HCC

were more likely than the females to have undergone blood transfusion (18.75% *vs* 8.33%, $P = 0.043$), consumed alcohol (31.25% *vs* 8.33%, $P = 0.010$), smoked (31.25% *vs* 8.33%, $P = 0.010$), had a family history of malignancy (25% *vs* 5.56%, $P = 0.012$), and had serious liver inflammation, as indicated by the elevated levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ -glutamyl transpeptidase ($P < 0.05$). Conditional logistic regression analysis revealed that body mass index (BMI) ≥ 25 [adjusted odds ratio (AOR) = 1.116, 95%CI: 1.002-1.244, $P = 0.045$] and history of alcohol intake (AOR = 10.294, 95%CI: 1.108-95.680, $P = 0.040$) were significantly associated with increased odds of HCC development in PBC patients.

CONCLUSION: HCC is not rare in Chinese PBC patients. Risk factors for PBC-associated HCC include BMI ≥ 25 and a history of alcohol intake. In addition to regular monitoring, PBC patients may benefit from abstinence from alcohol and body weight control.

Key words: Primary biliary cirrhosis; Hepatocellular carcinoma; Body mass index; History of alcohol intake; Case-control study

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Core tip: Previous studies have suggested that many factors are associated with hepatocellular carcinoma (HCC) development in primary biliary cirrhosis (PBC) patients. However, the evaluation of risk factors using a case-control study has not been reported. This case-control study analyzed the characteristics of PBC-associated HCC and investigated the relevant risk factors. The incidence of HCC was 4.13% in Chinese PBC patients, and it was more frequent in the male patients. Our results show for the first time that body mass index ≥ 25 and a history of alcohol intake are independent risk factors for HCC in PBC patients.

Zhang XX, Wang LF, Jin L, Li YY, Hao SL, Shi YC, Zeng QL, Li ZW, Zhang Z, Lau GKK, Wang FS. Primary biliary cirrhosis-associated hepatocellular carcinoma in Chinese patients: Incidence and risk factors. *World J Gastroenterol* 2015; 21(12): 3554-3563 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3554.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3554>

INTRODUCTION

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease that mainly affects middle-aged women. The disease is characterized by elevated alkaline phosphatase (ALP) levels, the presence of antimitochondrial antibodies, and the immune-mediated destruction of small-to-medium bile ducts

in the liver. PBC can result in chronic cholestasis and the eventual development of irreversible cirrhosis or hepatocellular carcinoma (HCC)^[1].

The clinical manifestations of PBC vary widely over the course of disease, and there are few treatment options. Increased awareness by clinicians and improved diagnostic techniques have enabled its diagnosis in a greater number of asymptomatic patients. However, the only drug approved by the United States Food and Drug Administration to treat PBC is ursodeoxycholic acid (UDCA), which has not been proven to satisfactorily lower mortality rates^[2]. Currently, liver transplantation is the only effective treatment for PBC patients who are in the terminal stage or have liver failure^[3].

In general, HCC only occurs at the end stages of liver diseases, and the most common risk factor is infection with hepatitis B virus (HBV) or hepatitis C virus (HCV)^[4,5]. Until recently, the development of HCC in PBC patients was considered rare^[6]. However, with early diagnosis and improved treatment, the survival time has increased along with the incidence of HCC in these patients, which now ranges from 0.76% to 5.9%^[7-12]. Although older age, male gender, advanced histological stage, and history of blood transfusion have been associated with significantly increased odds of HCC, no factors have been found to be directly correlated with HCC development in PBC patients^[7,13,14] other than comorbidities, such as HBV or HCV infection and portal hypertension^[13-15].

Most of the studies cited above were based on populations in Europe, the United States, or Japan. In China, the greater awareness of PBC among physicians and the application of new laboratory tests have revealed that this disease is not as rare as formerly thought, and a recent study shows that its prevalence rate is 492 cases per million in southern China^[16], but the national data is still lacking. Furthermore, there have been several studies concerning the diagnosis and treatment of HCC in PBC patients^[17-20]. However, little is specifically known about the epidemiology of PBC-associated HCC in Chinese population.

In this study, we retrospectively assessed 52 PBC patients with HCC out of 1255 PBC patients to clarify the incidence and clinical characteristics of PBC-associated HCC in China and explored potential risk factors.

MATERIALS AND METHODS

Data source and subjects

The Beijing 302 Hospital Research Ethics Committee approved the study protocol. This study involved an analysis of anonymous secondary data; therefore, no informed consent from patients was required.

Beijing 302 Hospital is the largest liver disease hospital in China. The hospital's clinical database holds records of the clinical histories, physical and laboratory

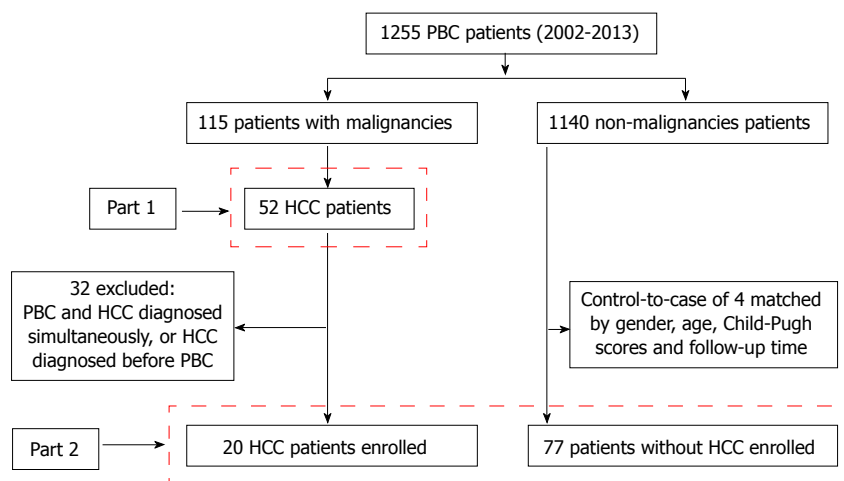


Figure 1 Derivation and definition of the study population. Part 1: Analysis of the characteristics of 52 patients with primary biliary cirrhosis (PBC)-associated hepatocellular carcinoma (HCC); Part 2: Analysis of potential risk factors for the development of HCC in PBC patients.

findings, and treatments of admitted patients, including those with viral hepatitis, autoimmune liver diseases, drug-induced hepatitis and inherited liver diseases. This database has been utilized previously in several studies^[21-23].

This was a retrospective study of patients diagnosed with PBC and treated at Beijing 302 Hospital between January 2002 and December 2013. The diagnosis of PBC was established when any two of the following three criteria were met: biochemical evidence of cholestasis (based mainly on ALP elevation); the presence of antimitochondrial autoantibodies; and histological evidence of non-suppurative destructive cholangitis and destruction of interlobular bile ducts^[24]. Patients were excluded who were HBV carriers, positive for HBV surface antigen (HBsAg) or anti-HCV antibody, or had autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), Wilson's disease, or α -1 antitrypsin deficiency.

This study comprised 1255 PBC patients, most of whom were receiving regular UDCA treatment. All patients were followed regularly, at least every 3-6 mo after hospital discharge, with biochemistry tests and abdominal sonography. A diagnosis of HCC was made based on imaging tests (magnetic resonance imaging with contrast media, computed tomography with contrast media or ultrasonic examination) or histological findings (surgery or biopsy)^[13]. During the study period, 52 cases of HCC were identified that met the above criteria for PBC and HCC.

A positive history of alcohol intake was defined as alcohol consumption at least once per week for at least 1 year (≥ 20 g/d for women and ≥ 30 g/d for men) without achieving the diagnostic criteria of alcoholic hepatitis^[13]. A positive history of smoking was defined as cigarette smoking for ≥ 4 d per week for at least 1 year^[24]. A family history of malignancy included one or more direct relatives suffering from any malignant tumor. Past HBV infection was defined as an HBsAg-

negative status and positive HBsAb and HBcAb statuses. Body mass index (BMI) was calculated as weight (in kilograms) divided by height squared (in meters).

Study design

We first determined the incidence of primary HCC in Chinese PBC patients. In addition, we reviewed the cases and collected the following laboratory data: alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP, γ -glutamyl transpeptidase (GGT), albumin, total bilirubin, and α -fetoprotein levels, prothrombin time, and white blood cell, red blood cell, and platelet counts. The main complaints (pruritus, fatigue, anorexia, and discomfort in the hepatic region), physical findings (jaundice and ascites) and medical history (blood transfusion, past HBV infection, alcohol intake, smoking, diabetes mellitus, hypertension, and family history of malignancy) were also recorded for these patients at the time of HCC diagnosis. The patients were stratified by gender for further statistical comparisons to analyze the characteristics between the two genders.

Gender and older age have been regarded as risk factors for PBC-associated HCC development^[13,14,26]. To determine other possible risk factors, we conducted a case-control study (Figure 1). Twenty patients diagnosed with PBC prior to HCC were identified. In addition, we selected PBC patients without HCC as controls, matching 4 controls to each PBC patient with HCC by age, gender, Child-Pugh scores and follow-up period. Because there were not enough matched control subjects for two cases, we used only two and three controls for these patients, respectively^[13]. We recorded the family history of malignancy, clinical and laboratory data, physical findings, and comorbidities for these patients at the time of PBC diagnosis.

Statistical analysis

The data are reported as the mean \pm SD or number

Table 1 Demographics and clinical features of primary biliary cirrhosis patients at hepatocellular carcinoma diagnosis *n* (%)

Variable	Total (<i>n</i> = 52)	Men (<i>n</i> = 16)	Women (<i>n</i> = 36)	<i>P</i> value ¹
Age (yr)	65.75 ± 10.09	69.75 ± 8.09	63.97 ± 10.47	0.093
Han nationality	47 (90.38)	14 (87.50)	33 (91.67)	0.638
Body mass index ≥ 25 (kg/m ²)	16 (30.77)	4 (25.00)	12 (33.33)	0.548
History of blood transfusion	6 (11.54)	3 (18.75)	3 (8.33)	0.043
Past HBV infection	29 (55.77)	10 (62.50)	19 (52.78)	0.519
Alcohol intake	8 (15.38)	5 (31.25)	3 (8.33)	0.035
Smoking	8 (15.38)	5 (31.25)	3 (8.33)	0.010
Type 2 diabetes mellitus	8 (15.38)	0	8 (22.22)	0.040
Hypertension	12 (23.08)	5 (31.25)	7 (19.44)	0.165
Family history of malignancy	6 (11.54)	4 (25.00)	2 (5.56)	0.012
Liver cirrhosis	52 (100.00)	16 (100.00)	36 (100.00)	1.000
Tumor size				
≥ 3 cm	35 (67.31)	11 (68.75)	24 (66.67)	0.882
< 3 cm	17 (32.69)	5 (31.25)	12 (33.33)	0.882
Tumor number				
Single	29 (55.77)	9 (56.25)	20 (55.56)	0.963
Multiple	23 (44.23)	7 (43.75)	16 (44.44)	0.963
Pruritus	4 (7.70)	3 (18.75)	1 (2.78)	0.046
Fatigue	37 (71.15)	13 (81.25)	24 (66.67)	0.284
Anorexia	20 (38.46)	5 (31.25)	15 (41.67)	0.476
Discomfort in hepatic region	11 (21.15)	4 (25.00)	7 (19.44)	0.651
Jaundice	30 (57.69)	11 (68.75)	19 (52.78)	0.282
Ascites	41 (78.85)	12 (75.00)	29 (80.56)	0.651

¹Men compared with women. HBV: Hepatitis B virus; NA: Not applicable.**Table 2** Biochemical characteristics of male and female primary biliary cirrhosis patients at hepatocellular carcinoma diagnosis

Variable	Total (<i>n</i> = 52)	Men (<i>n</i> = 16)	Women (<i>n</i> = 36)	<i>P</i> value ¹
Alanine transaminase (U/L)	43.04 ± 42.28	64.25 ± 55.48	33.61 ± 31.45	0.012
Aspartate aminotransferase (U/L)	77.83 ± 56.24	111.38 ± 72.47	62.92 ± 40.19	0.005
Alkaline phosphatase (U/L)	229.29 ± 190.44	308.81 ± 209.37	193.94 ± 172.87	0.015
γ-glutamyl transpeptidase (U/L)	156.90 ± 179.05	253.13 ± 220.54	114.14 ± 140.55	0.010
Albumin (g/L)	28.42 ± 5.67	28.06 ± 5.64	28.58 ± 5.75	0.720
Total bilirubin (μmol/dL)	61.19 ± 102.13	74.14 ± 64.25	55.43 ± 115.41	0.071
α-fetoprotein elevating, <i>n</i> (%)	34 (65.38)	9 (56.25)	25 (69.44)	0.356
Prothrombin time (s)	13.49 ± 2.20	12.79 ± 1.91	13.8 ± 2.28	0.159
International standardization ratio	1.15 ± 0.23	1.13 ± 0.14	1.17 ± 0.26	0.410
White blood cells (10 ⁹ /L)	4.29 ± 2.35	5.13 ± 2.32	3.92 ± 2.30	0.073
Red blood cells (10 ¹² /L)	3.31 ± 0.66	3.56 ± 0.76	3.19 ± 0.60	0.096
Platelets (10 ⁹ /L)	76.57 ± 40.50	85.20 ± 48.26	72.74 ± 36.64	0.506

¹Men compared with women. Data expressed as mean ± SD or *n* (%) of patients.

(percentage) of patients. The Mann-Whitney *U* and χ^2 tests were used as nonparametric and independent tests, respectively. Survival rates were obtained using the Kaplan-Meier method. Variables with a *P*-value < 0.1 were considered to be potential factors for the case-control study. Then, a conditional logistic regression model was used to estimate the relative magnitudes in relation to the potential factors. The odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated using patients without HCC as a reference. Analyses were performed using SPSS 16.0 software for Windows (SPSS, Chicago, IL, United States). All statistical tests were two-sided. A *P*-value < 0.05 was considered statistically significant.

RESULTS

The incidence and characteristics of PBC-associated HCC were identified in our database of 1255 PBC Chinese patients, which included 168 men and 1087 women. During nearly 12 years of follow-up, 115 patients were diagnosed with malignant tumors. There were 52 HCC patients, including 16 men and 36 women (Table 1). The overall HCC incidence was 4.13% (52/1255), and the incidence in men (9.52%) was almost three times higher than that in women (3.31%). The average follow-up period was 43.44 ± 39.91 mo for the entire group, with no statistically significant difference between the men (29.88 ± 22.84 mo) and

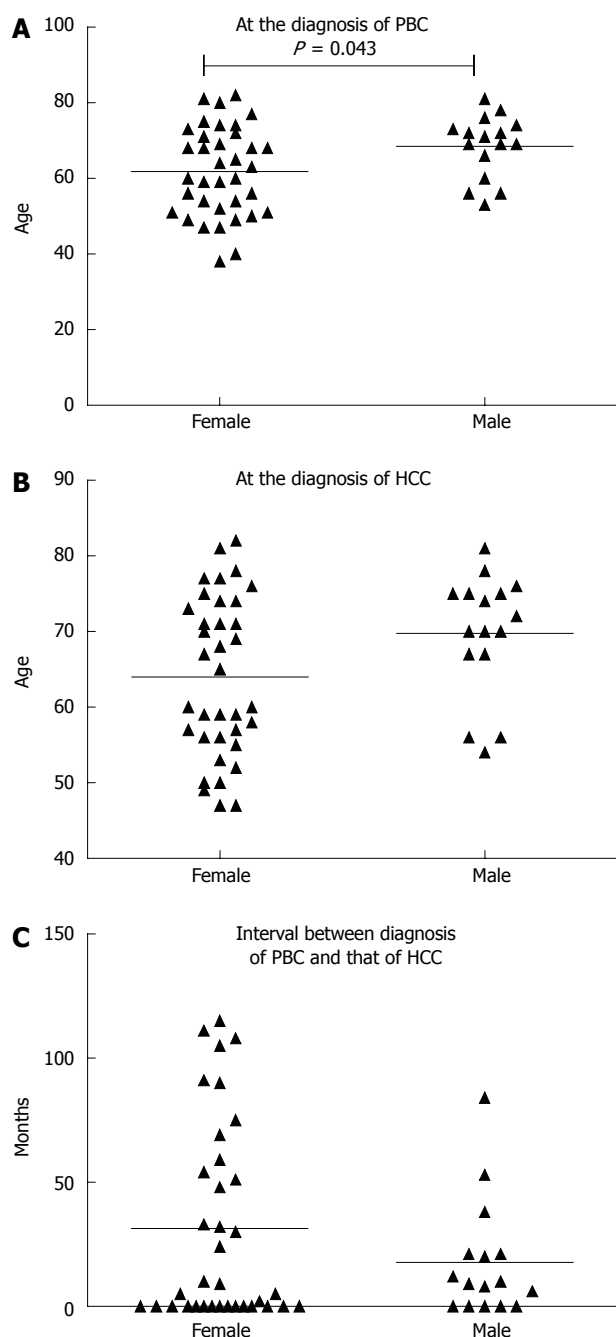


Figure 2 Average ages at the times of primary biliary cirrhosis diagnosis and hepatocellular carcinoma diagnosis, and intervals between these two diagnoses. The average age at primary biliary cirrhosis diagnosis was higher in men than in women ($P < 0.05$). Each triangle represents one patient. HCC: Hepatocellular carcinoma; PBC: Primary biliary cirrhosis.

women (49.47 ± 44.46 mo; $P = 0.346$).

Most of the 52 PBC patients with HCC were of Han nationality and were older than 60 years. Comparative analysis of the men and women showed that HCC prevalence was significantly higher among the men with a history of blood transfusion ($P = 0.043$), alcohol intake ($P = 0.035$), or smoking ($P = 0.010$) and a family history of malignancy ($P = 0.012$) (that is, one patient's mother had lung cancer, another patient's father and younger brother had gastric cancer, and one patient's father had HCC).

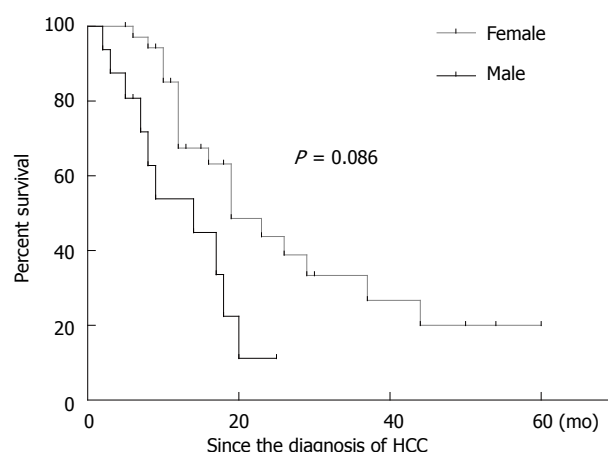


Figure 3 Kaplan-Meier curve for survival for the males and females with primary biliary cirrhosis-associated hepatocellular carcinoma. There was no statistically significant difference between the two genders ($P = 0.086$). HCC: Hepatocellular carcinoma.

More women than men had type-2 diabetes mellitus ($P = 0.040$).

All PBC patients were at the stage of liver cirrhosis when HCC was diagnosed. In most cases, there was a single tumor (55.77%), and the tumor size was more than 3 cm (67.31%). Portal venous thrombosis was present in five patients. Fatigue was the most common symptom (71.15%, 37/52), followed by anorexia (38.46%, 20/52) and discomfort in the hepatic region (21.15%, 11/52). Pruritus only occurred in four patients, including three men and one woman (18.75% vs 2.78%, $P = 0.046$). There were no significant differences in BMI, past HBV infection, history of hypertension, clinical manifestations, tumor size, or number of tumors between the two genders.

The serum levels of ALT, AST, ALP, and GGT were significantly higher in men than in women ($P < 0.05$ for all; Table 2). Approximately 65.38% of patients with PBC-associated HCC had elevated serum α -fetoprotein levels, but no significant differences were found between the men and women ($P = 0.356$).

Of the 52 PBC patients with HCC, 55.76% (29/52) received a diagnosis of HCC and PBC simultaneously, and 5.76% (3/52) were found to have HCC before being diagnosed with PBC. For the remaining 20 patients, HCC was diagnosed after PBC, and the characteristics of these patients were used for further studies. Upon diagnosis of PBC, the men were significantly older than the women (68.44 ± 8.23 years vs 61.78 ± 11.75 years, $P = 0.043$), and the men also tended to be older (69.75 ± 8.09 years) than the women (63.97 ± 10.47 years) at the time of HCC diagnosis, but this difference was not significant ($P = 0.056$). Regarding the interval between the diagnosis of PBC and the development of HCC, there was no statistically difference between the men (17.63 ± 23.15 mo) and women (31.27 ± 39.61 mo; $P = 0.21$; Figure 2). The prognosis of the men tended to be slightly poorer than that of the women, although this difference was not statistical significant ($P = 0.086$) (Figure 3).

Table 3 Demographic, clinical and biochemical characteristics of primary biliary cirrhosis patients with or without hepatocellular carcinoma at primary biliary cirrhosis diagnosis *n* (%)

Variable	PBC with HCC (<i>n</i> = 20)	PBC without HCC (<i>n</i> = 77)	<i>P</i> value
Follow-up (mo)	46.45 ± 41.85	57.20 ± 36.33	0.144
Male	8 (40)	30 (38.97)	1.000
Female	12 (60)	47 (61.04)	1.000
Age (yr)	61.05 ± 11.92	60.77 ± 11.52	0.808
Han nationality	18 (90.00)	73 (94.81)	1.000
Body mass index ≥ 25 (kg/m ²)	6 (30)	7 (9.10)	0.015
History of blood transfusion	0	4 (5.19)	0.300
Family history of malignancy	4 (20)	3 (3.90)	0.014
History of alcohol intake	7 (35)	10 (12.99)	0.022
History of smoking	4 (20)	6 (7.79)	0.112
History of type 2 diabetes mellitus	3 (15)	7 (9.09)	0.441
Liver cirrhosis	17 (85)	62 (79.22)	0.958
Hepatitis B core antibody	9 (45)	37 (48.05)	0.562
Child-Pugh, A/B/C (<i>n</i> / <i>n</i> / <i>n</i>)	8/11/1	32/42/3	0.900
Antimitochondrial autoantibodies			
(+)	18 (90)	65 (84.42)	0.529
(-)	2 (10)	12 (15.58)	0.529
IgA (mg/dL)	2.78 ± 1.32	3.13 ± 2.07	0.686
IgM (mg/dL)	3.46 ± 1.99	4.07 ± 3.34	0.482
IgG (mg/dL)	15.86 ± 3.24	15.55 ± 6.27	0.657
White blood cells (× 10 ⁹ /L)	4.14 ± 1.38	4.85 ± 2.52	0.449
Hemoglobin level (g/dL)	109.01 ± 16.28	108.42 ± 20.69	0.438
Alanine aminotransferase (U/L)	80.15 ± 63.50	75.91 ± 67.18	0.715
Aspartate aminotransferase (U/L)	97.5 ± 64.48	92.60 ± 72.85	0.611
Alkaline phosphatase (U/L)	278.25 ± 155.42	376.10 ± 433.46	0.918
γ-glutamyl transpeptidase level (U/L)	294.65 ± 285.01	316.29 ± 331.95	0.748
Total bilirubin level (mg/dL)	46.84 ± 64.34	46.19 ± 62.37	0.858
Albumin level (g/dL)	32.75 ± 6.20	33.21 ± 5.58	0.876
Triglyceride (mmol/L)	1.03 ± 0.43	1.36 ± 0.82	0.207
Total cholesterol (mmol/L)	4.20 ± 1.38	5.75 ± 4.05	0.113
Prothrombin time (s)	12.34 ± 1.64	12.23 ± 1.37	0.813
α-fetoprotein (ng/mL)	14.75 ± 21.84	7.62 ± 4.46	0.072
Use of ursodeoxycholic acid	19 (95)	60 (77.9)	0.080
Effective	8 (42.1)	23 (38.3)	0.769
Clinical stage			
Asymptomatic	0 (0)	9 (11.69)	0.110
Symptomatic	20 (100)	68 (88.31)	0.110

HCC: Hepatocellular carcinoma; PBC: Primary biliary cirrhosis.

Risk factors for HCC development in PBC patients

Of 52 patients with PBC and HCC, we selected 20 in whom PBC was diagnosed prior to HCC to analyze risk factors for HCC development. Using a case-control study design, records from these 20 cases and 77 matched controls (PBC patients without HCC) were analyzed to identify risk factors for the development of HCC in Chinese PBC patients (Table 3). Further assessments indicated that there were no significant differences in age, gender ratio, Child-Pugh scores or follow-up period between the two groups. However, BMI ≥ 25 ($P = 0.015$), family history of malignancy ($P = 0.014$), and history of alcohol intake ($P = 0.022$) significantly differed between the two groups, and in

further univariate analysis, these three factors were found to be associated with increases in crude ORs for HCC development (Table 4). There were no significant differences between the PBC-associated HCC cases and controls with regard to history of blood transfusion, history of smoking, diabetes mellitus, the levels of antimitochondrial autoantibodies, immunoglobulin, aminotransferase, α-fetoprotein, triglycerides or total cholesterol, prothrombin time, use of UDCA, or clinical stage. Adjustments for possible confounders (UDCA therapy and α-fetoprotein) only slightly altered the ORs. In the final model, BMI ≥ 25 [adjusted odds ratio (AOR) = 1.294; 95%CI, 1.054-1.589] and history of alcohol intake (AOR = 9.204; 95%CI, 1.019-83.129) were independent risk factors for HCC development in PBC patients (Table 4). However, BMI ≥ 25 and history of alcohol intake had no significant impact on the survival rate of patients with PBC-associated HCC ($P = 0.853$ and $P = 0.945$, respectively; Figure 4).

DISCUSSION

HCC often develops in patients with HBV or HCV infection, but its incidence has recently increased in patients with autoimmune liver diseases, including PBC, AIH, PSC, and overlap syndrome. The risks of hepatic and extrahepatic malignancies are significantly increased in patients with AIH and PSC, and response to therapy is often associated with prognosis^[27,28]. We conducted a retrospective study of patients treated at Beijing 302 Hospital between January 2002 and December 2013 to investigate the likelihood of developing HCC, its clinical features, and its related risk factors in Chinese PBC patients. We first found that HCC was present in 4.13% of PBC patients. The male PBC patients were more likely than the females to develop HCC, and it was more serious in the males. This is the first report that the risk factors BMI ≥ 25 and history of alcohol intake are independently associated with HCC development in Chinese PBC patients.

A previous meta-analysis has reported that the risk of HCC in PBC patients is more than 18.8-fold that of the general population^[29]. This finding indicates that it is necessary to screen PBC patients for HCC. The data from these two studies suggest that HCC is not as common in PBC patients as it is in those with other forms of cirrhosis^[8,30], while another study has reported conflicting results^[31]. This may be due to discrepancies in cirrhosis stages, follow-up periods or study samples. In 1997, Jones *et al*^[7] reported that the incidence of PBC-associated HCC in the United Kingdom was 5.9%, and their findings were based on 667 PBC patients who were followed for 20 years. However, a 9-year follow-up study of 1689 PBC patients in the United States by Angulo *et al*^[32] found that the incidence of PBC-associated HCC was 0.89%. Recently, Harada *et al*^[26] reported an incidence of 2.4% in Japan. In our study, we reported that its incidence in Chinese

Table 4 Univariate (unadjusted) and multivariate (adjusted) conditional logistic regression analysis of potential risk factors for hepatocellular carcinoma in primary biliary cirrhosis patients *n* (%)

Variable	Univariate OR		Multivariate OR	
	(95%CI)	<i>P</i> value	(95%CI)	<i>P</i> value
Body mass index ≥ 25 (kg/m ²)	3.819 (1.140-12.792)	0.006	1.116 (1.002-1.244)	0.045
Family history of malignancy	6.971 (1.253-38.790)	0.027	NA	0.175
History of alcohol intake	11.525 (1.338-99.280)	0.026	10.294 (1.108-95.680)	0.040
α-fetoprotein (ng/mL)	1.132 (1.016-1.260)	0.025	NA	0.058
Use of ursodeoxycholic acid (%)	78.828 (0.053-1.169 × 10 ⁵)	0.241	NA	0.159

OR: Odds ratio; NA: Not applicable.

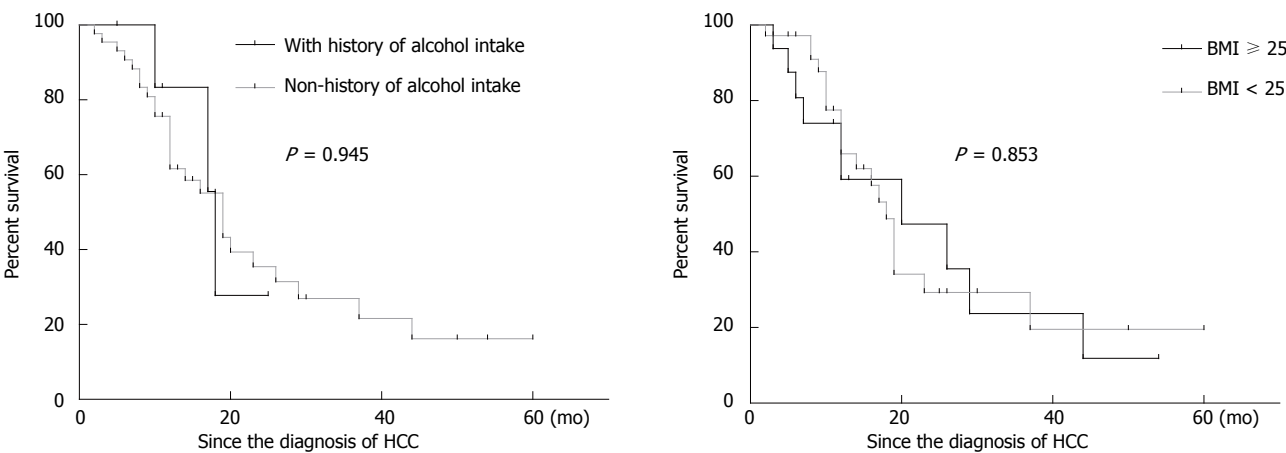


Figure 4 Kaplan-Meier curves for survival for the primary biliary cirrhosis patients with hepatocellular carcinoma. Patients were divided into two groups according to history of alcohol intake and body mass index. BMI: Body mass index.

PBC patients was 4.13%. This is in accord with other Chinese studies, which have reported values ranging from 2.57% in mainland China (350 cases followed for 5 years)^[33] to 5.2% in Taiwan (96 cases followed for 18 years)^[34]. However, the incidence might be underestimated in our paper, because some patients, who received liver transplantation, may develop HCC in their natural history. In addition, in the present study, approximately 60% of the patients received diagnoses of PBC and HCC simultaneously or of HCC prior to PBC, which is unique to China. These findings indicate that a lack of recognition of PBC still exists in China and that this condition should be taken seriously by the development of an intensive surveillance program.

In the present study, the incidence of HCC in the male PBC patients was 3-fold higher than that in the female patients. This finding is consistent with those of previous studies^[7,8,13-15]. These results are also in agreement with the finding that the severity of liver injury, as indirectly indicated by the liver function data, was more serious in male patients. Considering the effect of gender, we suggest that estrogen levels may have a role in PBC-associated HCC. Yeh *et al.*^[35] and Naugler *et al.*^[36] have found that estrogen can protect hepatocytes from malignant transformation, and male patients are more likely to develop HCC due to a lack of estrogen, although PBC mainly affects women. Moreover, some studies have shown that the absence

of the Y chromosome is associated with multiple malignant diseases^[37-39]. Unhealthy habits (such as alcohol drinking and cigarette smoking), history of blood transfusion, and family history of malignancy may also contribute to HCC development in male PBC patients^[26]. Above all, based on these studies, male PBC patients in particular should be screened carefully for HCC because of its higher incidence and severity in men. However, the exact reasons for these gender differences remain unknown and require further study to determine.

Studies of PBC-associated HCC have reported the involvement of some risk factors in its development, including male gender, blood transfusion, advanced histological stage, and age at PBC diagnosis^[7,8,13-15]. Results are not always congruent due to differences in sample sizes, follow-up periods, and methodologies. Based on prior studies, we conducted a case-control study of Chinese PBC patients with HCC compared with those without HCC, matching factors that included gender, age, follow-up time and Child-Pugh score^[40]. We enrolled 20 HCC cases with complete data at PBC diagnosis and HCC diagnosis. We first found that BMI ≥ 25 and history of alcohol intake were risk factors for PBC-associated HCC, which is consistent with previous studies^[41,42]. There were two studies showing that overweight status (BMI ≥ 25) was associated with advanced fibrosis in PBC patients^[25,43], indicating

that the immune reactions triggered by obesity and alcohol intake (even in small quantities) might contribute to PBC stage progression^[24]. Moreover, several studies have shown that obesity and history of alcohol intake synergistically increase the incidence of HCC by aggravating hepatic insulin resistance and necroinflammation in patients with HBV or HCV infection, HBV/HCV co-infection, or other liver diseases^[24,44,45]. Therefore, these two factors may promote HCC development in PBC patients. However, conflicting opinions remain concerning these two factors^[13,14], due to differences among ethnicities in the sensitivity to liver injury caused by alcohol^[46], the lack of a consistent definition of alcohol intake, and differences in sample sizes and follow-up time.

Several limitations of the present study should be noted. First, although histological stage at PBC diagnosis is a known important risk factor for HCC, we could not obtain this information because liver biopsies were not consistently performed. Second, UDCA treatment and α -fetoprotein level were not found to be risk factors for the development of PBC-associated HCC in our study. Racial factors may play a role, but basic studies of its underlying mechanisms are needed. Third, the retrospective nature of our study design may have made it difficult to obtain risk factors specific to PBC patients; thus, a further prospective study is warranted that should include more comprehensive groups. Fourth, there might have been an inclusion bias because only 20 cases met the inclusion criteria; thus, there may have been an insufficient number of patients evaluated to obtain adequate positive findings. Therefore, a study with a larger sample size should be performed.

In conclusion, HCC is not rare in Chinese patients with PBC. The incidence of PBC-associated HCC is higher and liver injury is more serious in men than in women. Because high BMI and alcohol intake are risk factors for HCC in PBC patients, these patients should be encouraged to stop alcohol consumption and control body weight.

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COMMENTS

Background

Previous studies have indicated that some risk factors are involved in hepatocellular carcinoma (HCC) development in primary biliary cirrhosis (PBC) patients. However, the evaluation of potential risk factors using a case-control study has not been reported.

Research frontiers

There is a lack of information regarding the risk factors for and incidence of PBC-associated HCC in China. Potential factors contributing to disease progression and death were included in this study to identify independent risk

factors for HCC development.

Related publications

This is the first case-control study to investigate risk factors for HCC development in PBC patients.

Innovations and breakthroughs

This study is the first case-control study to investigate risk factors for HCC development in PBC patients and the first to demonstrate that the incidence of PBC-associated HCC is 4.13% in the Chinese population. Analysis indicated that high BMI and alcohol intake might be risk factors for HCC development. Male PBC patients are more likely to develop HCC than females, and PBC-associated HCC in male patients is more severe than in female patients, as indicated by their high levels of alanine aminotransferase/aspartate aminotransferase/alkaline phosphatase/ γ -glutamyl transpeptidase.

Applications

The authors suggest that physicians should pay close attention to patients with PBC-associated HCC and advise PBC patients to stop drinking and control body weight. Male PBC patients should be screened carefully for HCC because of its high incidence and severity.

Peer-review

The authors investigated the cases and controls from their hospital clinical database to explore the incidence and risk factors for PBC-associated HCC and offer some valuable conclusions.

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Case Control Study

Hepatectomy with primary closure of common bile duct for hepatolithiasis combined with choledocholithiasis

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Abstract

AIM: To evaluate the feasibility of hepatectomy and primary closure of common bile duct for intrahepatic and extrahepatic calculi.

METHODS: From January 2008 to May 2013, anatomic hepatectomy followed by biliary tract exploration without biliary drainage (non-drainage group) was performed in 43 patients with intrahepatic and extrahepatic calculi. After hepatectomy, flexible choledochoscopy was used to extract residual stones and observe the intrahepatic bile duct and common bile duct (CBD) for determination of biliary stricture and dilatation. Function of the sphincter of Oddi was determined by manometry of the CBD. Primary closure of the CBD without T-tube drainage or bilioenteric anastomosis was performed when there was no biliary stricture or sphincter of Oddi dysfunction. Dexamethasone and anisodamine were intravenously injected 2-3 d after surgery to prevent postoperative retrograde infection due to intraoperative bile duct irrigation, and to maintain relaxation of the sphincter of Oddi, respectively. During the same period, anatomic hepatectomy followed by biliary tract exploration with biliary drainage (drainage group) was performed in 48 patients as the control group. Postoperative complications and hospital stay were compared between the two groups.

RESULTS: There was no operative mortality in either group of patients. Compared to intrahepatic and extrabiliary drainage, hepatectomy with primary closure of the CBD (non-drainage) did not increase the incidence

of complications, including residual stones, bile leakage, pancreatitis and cholangitis ($P > 0.05$). Postoperative hospital stay and costs were nevertheless significantly less in the non-drainage group than in the drainage group. The median postoperative hospital stay was shorter in the non-drainage group than in the drainage group (11.2 ± 2.8 d *vs* 15.4 ± 2.1 d, $P = 0.000$). The average postoperative cost of treatment was lower in the non-drainage group than in the drainage group (29325.6 ± 5668.2 yuan *vs* 32933.3 ± 6235.1 yuan, $P = 0.005$).

CONCLUSION: Hepatectomy followed by choledochoscopic stone extraction without biliary drainage is a safe and effective treatment of hepatolithiasis combined with choledocholithiasis.

Key words: Hepatolithiasis; Choledocholithiasis; Primary closure; Hepatectomy; Biliary drainage

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Core tip: We performed hepatectomy with primary closure of the common bile duct for hepatolithiasis combined with choledocholithiasis. Postoperative complications including residual stones, bile leakage, pancreatitis and cholangitis were equivalent in the drainage and non-drainage groups. Postoperative hospital stay and costs were nevertheless significantly less in the non-drainage than in the drainage group. Additional biliary drainage is not necessary for all patients with intrahepatic and extrahepatic calculi, thus avoiding unnecessary discomfort and extra costs. Anatomic hepatectomy followed by intraoperative choledochoscopic stone extraction without biliary drainage in selected patients is a safe and effective treatment.

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INTRODUCTION

Intrahepatic and extrahepatic lithiasis is a common disease in Asia, especially in East and Southeast Asia. Calculi, biliary infection, biliary stricture and hepatic parenchymal fibrosis make it a complicated and intractable clinical problem. Various treatments have been proposed for intrahepatic and extrahepatic lithiasis; however, no consensus has been reached on the ideal treatment. Surgery achieves good results with low morbidity and mortality^[1,2]. Potential operations for intrahepatic and extrahepatic lithiasis include: (1) hepatectomy; (2) extraction of calculi *via*

choledochotomy; and (3) placement of a T-tube or creation of a bilioenteric anastomosis^[1]. Hepatectomy of the affected segment, including intrahepatic calculi and potential biliary stenoses, is probably the best therapeutic option, because it achieves the best long-term results^[3-5].

Traditionally, after resection of hepatic lesions and choledochotomy, the common bile duct (CBD) is drained with a T-tube for choledocholithiasis combined with hepatolithiasis. However, insertion of a T-tube is related to some potential postoperative complications. Some of these complications are serious, such as bile leak, biliary tract infection, or acute renal failure from dehydration due to inadequate water ingestion or a high outflow. In addition, patients must carry the T-tube for several weeks before its removal, causing significant discomfort^[6-8]. Choledochoduodenostomy has been performed at many hospitals for many years worldwide, and is considered a safe and effective method for treatment of choledocholithiasis^[9-15]. However, the use of choledochoduodenostomy for treatment of hepatolithiasis has been controversial for a long time^[16,17]. Choledochoduodenostomy does not entirely achieve the goal of stone clearance, correction of strictures, and removal of hepatobiliary lesions by itself, thus, there is no definitive evidence to show the long-term outcomes of choledochoduodenostomy for hepatolithiasis. Moreover, choledochoduodenostomy without cholangioplasty results in an increase of severe reflux cholangitis due to the loss of the anti-reflux function of the sphincter of Oddi. Therefore, choledochoduodenostomy is not an ideal approach to reduce cholangitis in hepatolithiasis and is not the best choice in the management of hepatolithiasis^[18,19]. Hepaticojejunostomy, another drainage modality, is also an imperfect solution due to recurrence of symptoms. Herman *et al*^[20] found that 41.2% of patients who underwent liver resection associated with hepaticojejunostomy had late postoperative complications such as cholangitis or liver abscess during follow-up. Similar to choledochoduodenostomy and hepaticojejunostomy, choledochojejunostomy is used to drain fluid in patients with hepatolithiasis complicated with intrahepatic biliary stricture after partial hepatectomy and hilar cholangioplasty. However, loss of the sphincter of Oddi, biliary reflux, and gastrointestinal dysfunction often occur after traditional choledochojejunostomy^[16,21,22]. Finally, the rate of cholangiocarcinoma is related to that of cholangitis, which occurs in choledochoduodenostomy and hepaticojejunostomy^[23], making bilioenteric anastomosis a controversial procedure.

We have performed a retrospective study of hepatectomy with primary closure of the CBD or biliary drainage for treatment of hepatolithiasis combined with choledocholithiasis. The purpose of the present study was to evaluate the feasibility and efficacy of hepatectomy and primary closure of the CBD for intrahepatic and extrahepatic lithiasis.

Table 1 Calculus distribution, liver lesions and operations

Calculus distribution and liver lesions	Operations	
	Drainage group (<i>n</i> = 48)	Non-drainage group (<i>n</i> = 43)
Left lobe stones, parenchymal atrophy and/or fibrosis	Left hemihepatectomy, T-tube drainage (12) Left lateral segmentectomy, T-tube drainage (10) Left hemihepatectomy, choledochojejunostomy (3)	Left hemihepatectomy (12) Left lateral segmentectomy (8)
Right lobe stones, parenchymal atrophy and/or fibrosis	Right hemihepatectomy, T-tube drainage (6) Right posterior lobectomy, T-tube drainage (4) Right hemihepatectomy, hepaticojejunostomy (2)	Right hemihepatectomy (6) Right posterior lobectomy (3)
Bilateral lobe stones, dominantly affected left lobe atrophy and/or fibrosis	Left hemihepatectomy, right intrahepatic duct stone extraction, T-tube drainage (2) Left lateral segmentectomy, right intrahepatic duct stone extraction, T-tube drainage (3) Left hemihepatectomy, right intrahepatic duct stone extraction, choledochojejunostomy (2)	Left hemihepatectomy, right intrahepatic duct stone extraction (4) Left lateral segmentectomy, right intrahepatic duct stone extraction (6)
Bilateral lobe stones, dominantly affected right lobe atrophy and/or fibrosis	Right hemihepatectomy, left intrahepatic duct stone extraction, T-tube drainage (1) Right hemihepatectomy, left intrahepatic duct stone extraction, hepaticojejunostomy (2) Right hemihepatectomy, left intrahepatic duct stone extraction, choledochojejunostomy (1)	Right hemihepatectomy, left intrahepatic duct stone extraction (4)

Apart from intrahepatic lithiasis, all patients have extrahepatic bile duct stones.

MATERIALS AND METHODS

Patient data

From January 2008 to May 2013, 91 patients diagnosed with intrahepatic and extrahepatic lithiasis underwent hepatectomy and biliary tract exploration with or without biliary drainage in our department. Patients were divided into two groups: one that underwent anatomic hepatectomy and biliary tract exploration with biliary internal or external drainage (drainage group, *n* = 48); and another that underwent anatomic hepatectomy and biliary tract exploration without any biliary drainage (non-drainage group, *n* = 43).

There were 18 men (37.5%) and 30 women (62.5%), with a mean age of 54.8 years (range: 23-68 years) in the drainage group. There were 17 men (39.5%) and 26 women (60.5%), with a mean age of 59.3 years (range: 28-71 years) in the non-drainage group. The main symptoms of patients with hepatolithiasis were abdominal pain, fever, and jaundice. A history of right upper quadrant pain was present in all cases, and jaundice in 20 patients (41.7%) in the drainage group and 17 (39.5%) in the non-drainage group.

All cases were evaluated with routine investigations including full blood counts, liver function tests, and coagulation screening. Preoperative diagnosis was based on clinical presentation and imaging technologies, such as ultrasonography, spiral three-phase computed tomography, endoscopic retrograde cholangiopancreatography, and magnetic resonance cholangiography. Indications for liver resection were parenchymal atrophy, intrahepatic biliary stenosis and unilobular severe liver fibrosis.

Surgical procedure

All patients underwent cholecystectomy, except

16 patients who had previous cholecystectomy. Anatomic hepatectomy was performed in all patients in both groups. The CBD was opened through a supraduodenal vertical incision. Stones in the CBD and residual liver were removed by saline flushing. Flexible choledochoscopy was used to extract residual stones and observe the intrahepatic and extrahepatic bile ducts for determination of biliary stricture and dilatation. Function of the sphincter of Oddi was determined by manometry of the CBD in the non-drainage group. If there were no intrahepatic and extrahepatic residual stones, no extrahepatic biliary stricture, and the pressure of CBD was < 12 cmH₂O, primary closure of the CBD without biliary drainage was performed in the non-drainage group. Interrupted 4/0 or 5/0 Prolene sutures were used to complete primary closure of the CBD. In the non-drainage group, dexamethasone and anisodamine were intravenously injected 2-3 d after the operation to prevent postoperative retrograde infection due to intraoperative bile duct irrigation, and to maintain relaxation of the sphincter of Oddi, respectively. In the drainage group, T-tube drainage or creation of a bilioenteric anastomosis was performed after hepatectomy and biliary tract exploration. A sub-hepatic drainage tube was placed in all patients in both groups. T-tube cholangiography was performed on postoperative day 10 in all T-tube-drained patients. Once CBD clearance was confirmed and there was a free flow of contrast agent, the T-tube was removed 4 wk after the operation. Liver function was re-examined 3 and 7 d after the operation and computed tomography or ultrasound B detection was performed before discharge. Calculus distribution, liver lesions and operations are presented in Table 1. If no postoperative complications occurred, patients were discharged once the peritoneal drain was removed and the incision in the abdominal wall healed. Postoperative complications

Table 2 Postoperative complications, hospital stay and costs *n* (%)

Complication	Drainage group (<i>n</i> = 48)	Non-drainage group (<i>n</i> = 43)	<i>P</i> value
Residual stones	7 (14.6)	4 (9.3)	0.329
Bile leakage	7 (14.6)	6 (13.9)	0.586
Pancreatitis	2 (4.2)	2 (4.7)	0.649
Cholangitis	13 (27.1)	9 (20.9)	0.331
T-tube dislocation	1 (2.1)	0	0.527
Postoperative hospital stay (d)	15.4 ± 2.1	11.2 ± 2.8	0.000
Postoperative costs (yuan)	32 933.3 ± 6235.1	29 325.6 ± 5668.2	0.005

Postoperative hospital stay and costs were tested by Student's *t* test. Other variables were tested by Fisher's exact test or χ^2 test.

including bile leakage, jaundice, cholangitis, pancreatitis and T-tube dislocation were observed and compared between the two groups. Bile leakage was defined as bilirubin concentration in the drain fluid at least three times the serum bilirubin concentration on or after postoperative day 3, or as the need for radiological or operative intervention resulting from biliary collection or bile peritonitis^[24]. Diagnosis of cholangitis was made by the presence of abdominal pain, jaundice and/or fever. Hospital stay and costs varied from one patient to another in that different patients received different medication due to different preoperative conditions. Postoperative hospital stay and costs were only affected by the modality of operation and clinical outcome of individuals, thus directly reflecting the different values of different operations between the two groups. Parameters of postoperative hospital stay and costs were used in this study.

All patients received regular follow-up assessments every 6 mo. Mean follow-up was 35.3 mo, ranging from 12 to 64 mo.

Statistical analysis

Continuous variables are presented as the mean ± SD. The data were analyzed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, United States). Fisher's exact test or χ^2 was used for categorical variables to calculate frequencies and percentages among the groups. Student's *t* test was applied for continuous variables to compare the means (two-tailed) with median and range among the groups. *P* < 0.05 was considered statistically significant.

RESULTS

There were no significant differences in the demographic characteristics and clinical presentations between the two groups. The surgical methods in both groups are presented in Table 1. There was no surgical mortality in either group. Postoperative complications and hospital stay are compared between the two groups in Table 2. Compared to intrahepatic and extrabiliary

drainage, hepatectomy with primary closure of the CBD (non-drainage) did not increase the incidence of complications, including residual stones, bile leakage, pancreatitis and cholangitis. Seven cases (14.6%) of residual stones were found in the drainage group and four cases (9.3%) in the non-drainage group. Bile leakage was found in seven patients (14.6%) in the drainage group and six (13.9%) in the non-drainage group. In the drainage group, there was one case of T-tube dislocation 10 d after the operation, which responded well to conservative treatment. Transient acute pancreatitis developed in two patients (4.2%) in the drainage group and two (4.7%) in the non-drainage group. Of the two pancreatitis patients in the non-drainage group, one had pancreatitis combined with pancreatic abscess that required percutaneous drain insertion for 2 wk. Cholangitis was found in 13 patients (27.1%) in the drainage group and nine (20.9%) in the non-drainage group. As shown in Table 2, median postoperative hospital stay was shorter in the non-drainage group than in the drainage group (11.2 ± 2.8 d vs 15.4 ± 2.1 d, *P* = 0.000). The average postoperative cost of treatment was lower in the non-drainage group than in the drainage group (29 325.6 ± 5668.2 yuan vs 32 933.3 ± 6235.1 yuan, *P* = 0.005).

DISCUSSION

Choledochotomy followed by T-tube drainage is a traditional surgical treatment for extrahepatic lithiasis^[25,26]. Recently, primary closure of the CBD has been proposed as a safe alternative to T-tube placement after both laparoscopic choledochotomy and open choledochotomy in extrahepatic lithiasis^[27]. Therefore, the occurrence of postoperative complications related to T-tube placement, such as bile leak, biliary tract infection, dehydration and electrolyte disturbance has been significantly reduced. The patients without T-tube drainage feel more comfortable than those with the T-tube. Apart from T-tube drainage, bilioenteric anastomosis has also been considered a safe and effective method for the treatment of choledocholithiasis and hepatolithiasis^[9,28]. However, biliary reflux, and gastrointestinal dysfunction often occur after choledochojejunostomy and hepaticojejunostomy^[13,20,21,29]. Recurrence of symptoms in patients with choledochojejunostomy and hepaticojejunostomy showed that this may not be the ideal solution for both intrahepatic and extrahepatic lithiasis. Some studies have evaluated the long-term results of liver resection with or without hepaticojejunostomy for the treatment of primary intrahepatic lithiasis^[20]. However, so far, there have been no attempts to evaluate the therapeutic impact of hepatectomy and biliary tract exploration without biliary drainage on intrahepatic lithiasis combined with extrahepatic lithiasis. We performed a retrospective study of hepatectomy and biliary tract exploration without biliary internal or external

drainage for treatment of hepatolithiasis combined with choledocholithiasis. The patients recovered well if there were no residual stones, no extrahepatic biliary stricture, and ≤ 12 cmH₂O pressure in the CBD. Postoperative complications including residual stones, bile leakage, pancreatitis and cholangitis were equivalent in the drainage and non-drainage groups. Postoperative hospital stay and costs were significantly less in the non-drainage group than in the drainage group. Additional biliary drainage was not necessary for all patients with intrahepatic and extrahepatic calculi, thus avoiding unnecessary discomfort and extra costs.

The principles of definitive surgery for hepatolithiasis comprise complete removal of lesions, establishment of satisfactory drainage of the affected segments of the biliary tree and prevention of recurrence^[30,31]. Here, removal of lesions plays a crucial role in treatment of hepatolithiasis. The lesions of hepatolithiasis include stones, stricture, dilation, and affected hepatic tissues. Hepatectomy, especially anatomic hepatectomy, is the optimized method for this condition, which completely removes diseased bile duct and its drainage area, thus reducing the risk of long-term recurrence of stones, and may also prevent the complication of cholangiocarcinoma^[13,15,31-37]. Anatomic hepatectomy should be considered as first-line treatment of regional hepatolithiasis.

We performed anatomic hepatectomy and biliary tract exploration, thus completely removing intrahepatic and extrahepatic stones, bile duct stricture, and atrophic or fibrous liver lesions. We explored the biliary tract using choledochoscopy and determined the function of the sphincter of Oddi by manometry of the CBD. Primary closure of the CBD without any biliary drainage was only performed in circumstances in which intrahepatic and extrahepatic stones were completely removed, extrahepatic biliary stricture did not exist, and the pressure of CBD was in the normal range. We measured the pressure of CBD using a scale-marked transparent tube. If the function of the sphincter of Oddi was normal and drained in an unobstructed manner, the liquid level inside the tube decreased smoothly and finally fluctuated in the normal range. The sphincter of Oddi function was preserved so that the rate of postoperative recurrence of cholangitis was lower than in patients without preservation of sphincter of Oddi function. Other studies have also indicated that long-term results of procedures that preserve the sphincter of Oddi have lower rates of postoperative recurrent cholangitis than those without preservation of the sphincter of Oddi^[10,19,38].

In the non-drainage group in our study, patients with and without dilated CBD recovered uneventfully. So the CBD diameter is not a prerequisite for the operation without biliary drainage, in that normal CBD diameter might not increase the risk of bile duct stricture and bile leakage. On the contrary, normal CBD diameter implies normal papillary function and an unobstructed distal segment of the CBD. Compared to

the diameter of the CBD, sufficient blood supply to the CBD is more important in preventing postoperative bile duct stricture and bile leakage. Furthermore, in the non-drainage group, dexamethasone and anisodamine were intravenously injected 2-3 d after the operation. Hence, postoperative papillary edema was alleviated and the sphincter of Oddi remained relaxed to some extent, thus guaranteeing surgical success. In the non-drainage group, patients with preoperative jaundice recovered uneventfully. The rate of postoperative leakage and jaundice in the non-drainage group was similar to that in the drainage group. Thus, we conclude that drainage is not necessary if jaundice can be determined as obstructive jaundice due to extrahepatic stones. Considering the residual stones, we should be cautious to perform such operation in patients with multiple stones in both lobes of the liver. If unilateral intrahepatic stones, and atrophic or fibrous lesions of the liver are completely removed by hepatectomy, and the contralateral intrahepatic stones are completely extracted by choledochoscopy, hepatectomy with primary closure of the CBD can still be performed.

COMMENTS

Background

Surgical treatment achieves good results for intrahepatic and extrahepatic lithiasis. However, to date, there have been no attempts to evaluate the therapeutic impact of hepatectomy and biliary tract exploration without biliary drainage on intrahepatic lithiasis combined with extrahepatic lithiasis.

Research frontiers

This study showed that hepatectomy followed by choledochoscopic stone extraction without biliary drainage in selected patients is safe and effective for treatment of hepatolithiasis combined with choledocholithiasis. Additional biliary drainage is not necessary for all patients with intrahepatic and extrahepatic calculi, thus avoiding unnecessary discomfort and extra costs.

Innovations and breakthroughs

After hepatectomy, the authors measured common bile duct (CBD) pressure using a scale-marked transparent tube. If sphincter of Oddi function was normal with unobstructed drainage, the liquid level inside the tube decreased smoothly and finally fluctuated within the normal range. Dexamethasone and anisodamine were intravenously injected 2-3 d postoperatively to prevent retrograde infection due to intraoperative bile duct irrigation, and to maintain relaxation of the sphincter of Oddi, respectively. Hence, postoperative papillary edema was alleviated and sphincter of Oddi was maintained in a state of relaxation, thus guaranteeing surgical success.

Applications

Jia CK *et al* performed hepatectomy and biliary tract exploration without biliary internal or external drainage for treatment of hepatolithiasis combined with choledocholithiasis. Patients recovered well if there were no residual stones, no extrahepatic biliary stricture, and ≤ 12 cmH₂O CBD pressure. Postoperative hospital stay and costs were significantly less in the non-drainage group than in the drainage group. Additional biliary drainage is not necessary for all patients with intrahepatic and extrahepatic calculi, thus avoiding unnecessary discomfort and extra costs.

Terminology

Hepatectomy followed by choledochoscopic stone extraction without biliary drainage in selected patients is safe and effective for treatment of hepatolithiasis combined with choledocholithiasis.

Peer-review

This is a retrospective case-control study comparing treatments of intrahepatic stones with extrahepatic components by hepatectomy and CBD exploration with or without drainage.

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Retrospective Study

Thiopurine metabolites variations during co-treatment with aminosalicylates for inflammatory bowel disease: Effect of N-acetyl transferase polymorphisms

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Abstract

AIM: To evaluate variation of the concentration of thiopurine metabolites after 5-aminosalicylate (5-ASA) interruption and the role of genetic polymorphisms of N-acetyl transferase (NAT) 1 and 2.

METHODS: Concentrations of thioguanine nucleotides (TGN) and methymercaptopurine nucleotides (MMPN), metabolites of thiopurines, were measured by high performance liquid chromatography in 12 young patients (3 females and 9 males, median age 16 years) with inflammatory bowel disease (6 Crohn's disease and 6 ulcerative colitis) treated with thiopurines (7 mercaptopurine and 5 azathioprine) and 5-ASA. Blood samples were collected one month before and one month after the interruption of 5-ASA. DNA was extracted and genotyping of *NAT1*, *NAT2*, inosine triphosphate pyrophosphatase (*ITPA*) and thiopurine methyl transferase (*TPMT*) genes was performed using PCR assays.

RESULTS: Median TGN concentration before 5-ASA interruption was 270 pmol/8 x 10⁸ erythrocytes (range: 145-750); after the interruption of the aminosalicylate, a 35% reduction in TGN mean concentrations (absolute

mean reduction $109 \text{ pmol}/8 \times 10^8$ erythrocytes) was observed (median $221 \text{ pmol}/8 \times 10^8$ erythrocytes, range: 96-427, *P* value linear mixed effects model 0.0011). Demographic and clinical covariates were not related to thiopurine metabolites concentrations. All patients were wild-type for the most relevant ITPA and TPMT variants. For NAT1 genotyping, 7 subjects presented an allele combination corresponding to fast enzymatic activity and 5 to slow activity. NAT1 genotypes corresponding to fast enzymatic activity were associated with reduced TGN concentration (*P* value linear mixed effects model 0.033), putatively because of increased 5-ASA inactivation and consequent reduced inhibition of thiopurine metabolism. The effect of NAT1 status on TGN seems to be persistent even after one month since the interruption of the aminosaliclate. No effect of NAT1 genotypes was shown on MMPN concentrations. NAT2 genotyping revealed that 6 patients presented a genotype corresponding to fast enzymatic activity and 6 to slow activity; NAT2 genotypes were not related to thiopurine metabolites concentration in this study.

CONCLUSION: NAT1 genotype affects TGN levels in patients treated with thiopurines and aminosaliclates and could therefore influence the toxicity and efficacy of these drugs; however the number of patients evaluated is limited and this has to be considered a pilot study.

Key words: Thiopurines; Aminosaliclates; Inflammatory bowel diseases; N-acetyl transferase; Pharmacogenomics

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Core tip: During treatment of inflammatory bowel disease with thiopurines and aminosaliclates, interruption of the aminosaliclate results in a significant decrease in thiopurines' thioguanine nucleotides (TGN) active metabolites. Genetic polymorphisms in genes involved in aminosaliclates biotransformation (NAT1 genotype) affects TGN levels in patients treated with thiopurines and aminosaliclates and could therefore influence the toxicity and efficacy of these drugs.

Stocco G, Cuzzoni E, De Iudicibus S, Favretto D, Malusà N, Martelossi S, Pozzi E, Lionetti P, Ventura A, Decorti G. Thiopurine metabolites variations during co-treatment with aminosaliclates for inflammatory bowel disease: Effect of N-acetyl transferase polymorphisms. *World J Gastroenterol* 2015; 21(12): 3571-3578 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3571.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3571>

INTRODUCTION

Thiopurines and aminosaliclates are the two most widely used drugs in inflammatory bowel disease (IBD)

and are often used in combination. The thiopurines 6-mercaptopurine (6MP) and its prodrug azathioprine (AZA) are effective in inducing and maintaining remission and are considered steroid sparing agents. 6MP is metabolized by a multistep enzymatic pathway, initiated by hypoxanthine phosphoribosyl transferase that leads to formation of thioguanine nucleotides (TGNs). These active metabolites act as purine antagonist and inhibit DNA, RNA and protein synthesis, inducing cytotoxicity and immunosuppression. Blood levels of thiopurine metabolites have been correlated with the efficacy and toxicity of these drugs in patients with IBD: TGN levels higher than $235 \text{ pmol}/8 \times 10^8$ red blood cells are considered therapeutic, and methyl mercaptopurine nucleotides (MMPNs) levels above $5700 \text{ pmol}/8 \times 10^8$ red blood cells have been associated with hepatotoxicity^[1-3].

The aminosaliclate 5-aminosalicylic acid (mesalazine, 5-ASA) is used in the induction and maintenance of remission in ulcerative colitis^[4,5]. In Crohn's disease, the use of aminosaliclates is controversial, however studies suggest that they could have a role in the postoperative maintenance of remission also in this IBD^[6]. In addition, a chemopreventive role of 5-ASA in IBD against colon cancer has been suggested^[7].

An increase in mean TGN blood levels has been reported in patients on 6MP or AZA co-treated with 5-ASA^[2,8-12]. Even more important, a higher rate of myelotoxicity was observed in patients treated with this combination in comparison with those treated with the thiopurine alone^[2,9,13].

6MP is inactivated by the enzyme thiopurine methyltransferase (TPMT, EC 2.1.1.67) that catalyzes its S-methylation to 6-methylmercaptopurine and, at least in part by inosine triphosphate pyrophosphatase (ITPA, EC 3.6.1.19). *In vitro* studies have shown that aminosaliclates and their metabolites can inhibit the activity of TPMT^[14,15], however, this observation has not been confirmed *in vivo*^[2,8,9].

The enzymes N-acetyltransferases (NAT1 and NAT2, EC 2.3.1.5) are responsible for the N-acetylation of a number of xenobiotics and drugs including the aminosaliclates. Even the activity of NAT1 and NAT2 is genetically determined and subjects are classified as rapid, intermediate or slow acetylators. Although NAT1 and NAT2 polymorphisms have been associated with the incidence of some diseases, no significant effect has been reported for IBD^[16]. 5-ASA is inactivated primarily by the NAT1 isoform in the colonic mucosa, and the drug and its metabolites are excreted in the urine^[17-19]. The inheritance of a slow acetylator genotype for NAT1 could therefore lead to a reduced inactivation of 5-ASA, and hence to higher blood levels of the drug.

The aim of this study was to measure variation of the concentration of thiopurine metabolites after 5-ASA interruption and to evaluate the role of genetic polymorphisms of NAT1 and NAT2 on this phenomenon.

Table 1 Genotypes and methods of analysis considered in this study

Gene	Polymorphism	Method	References
NAT1	T1088A	Sequencing	With primer forward: 5'-TGCCC AAACATGGTGATAGATT-3' With primer reverse: 5'-CCATAA AACTTTCTAGGAATTCACA AT-3'
NAT1	C1095A	Sequencing	As above
NAT2	C282T	PCR-RFLP	[23,24]
NAT2	T341C	PCR-RFLP	[23,24]
TPMT	G238C	PCR-ASO	[27]
TPMT	G460A	PCR-RFLP	[27]
TPMT	A719G	PCR-RFLP	[27]
ITPA	C94A	TaqMan	TaqMan SNP genotyping assay from Applied biosystems (C_27465000_10)

MATERIALS AND METHODS

Patients and inclusion criteria

Twelve patients with IBD were enrolled by the Gastroenterology Unit of the Pediatric Hospital "Burlo Garofolo" in Trieste, and by the Research Children's Hospital "Meyer", Florence, Italy. These patients have been retrospectively selected considering the following criteria: previous diagnosis of IBD and treatment with AZA or 6MP plus 5-ASA for at least three months. 5-ASA therapy was interrupted, and a minimum of two blood samples for thiopurine metabolites measurement were taken one month before and one month after 5-ASA interruption. The study was approved by the local ethical committees and appropriate informed consent was obtained from all patients or their parents or guardians.

Measurement of azathioprine metabolites

Azathioprine metabolites (TGN and MMPN) were measured in patients' erythrocytes using an HPLC assay by Dervieux and Bouliou^[20] within few weeks from the sample collection. The ratio between TGN and the dose of azathioprine was calculated to account for the respective dose each patient was taking on the day that the metabolite testing was performed.

Genotypes

Genomic DNA was extracted from peripheral blood samples using a commercial kit (SIGMA, Milan, Italy), in order to characterize genetic polymorphisms in the candidate genes *NAT1*, *NAT2*, *TPMT* and *ITPA*. The considered genotypes and method of analysis are described in Table 1.

NAT acetylator status determination

NAT acetylator status (*i.e.*, rapid or slow) was assessed from the genotyping results. In particular, for *NAT1*, patients with an A nucleotide at both 1088 and 1095 nucleotides, corresponding to *NAT1**10 allele, were

considered as fast *NAT1* acetylators while all other genotype combinations were considered as slow *NAT1* acetylators^[21,22]. For *NAT2*, patients homozygous for the wild-type allele at either the 282 or 341 position or patients heterozygous for the variant allele at just one of these two positions were considered as fast *NAT2* acetylators, all other genotype combinations were considered as slow *NAT2* acetylators^[23,24].

Statistical analysis

Statistical analysis was performed using the software R (version 3.0.1).

The primary intended outcome of this study was to evaluate variations of the concentration of thiopurine metabolites after 5-ASA interruption and the role of genetic polymorphisms of *NAT 1* and *2*.

Power analyses on preliminary data available indicate that given the difference in means and the distribution's standard deviation, the minimum sample size to identify a statistically significant ($P = 0.05$, power 80%) result is 9 for the paired test comparing azathioprine metabolites during aminosalicylate treatment and after the suspension. For the analysis comparing thiopurine metabolites concentration in *NAT1* fast acetylators compared to slow acetylators, the minimum number of patients to detect a statistically significant ($P = 0.05$, power 80%) result is 5 for each *NAT1* activity status.

The association between pharmacological phenotypes of interest (*i.e.*, TGN metabolites concentrations, MMPN metabolites concentrations) and the considered demographic variables, IBD type, co-treatment with aminosalicylate or genotypes in a univariate analysis, was evaluated by considering for each phenotype and patient the individual observations and evaluating the effect of each covariate by calculating the P value from a linear mixed effects model built using the phenotype as the dependent variable, each covariate as the fixed effect and the patients as the random effect in the model. Multivariate analysis was done to test the independence of the effects of the covariates significant in the univariate analysis on the phenotypes considered by using linear mixed effects models with the phenotype of interest as the independent variables and the covariates selected in the univariate analysis as the dependent variables. For all parametric analyses (*i.e.*, linear mixed effects models used in the univariate analysis and the multivariate analysis), normality of the phenotype was tested by the Shapiro test and log10 transformation was applied if needed, in order to achieve normality of the distribution.

RESULTS

Patients enrolled and samples collected

The present study recruited 12 young patients (3 females and 9 males, median age 16 years) with IBD (6 Crohn's disease and 6 ulcerative colitis). Seven

Table 2 Demographic, clinical and pharmacological data for the 12 patients enrolled

Patient	Age at enrollment (yr)	Disease	Thiopurine dose (mg/kg per day)	5-ASA dose (mg/d)	TGN concentration ¹		% TGN change	NAT1 status
					Before	After		
1	7.7	CD	AZA 2.6	50	244	218	-11%	Rapid
2	17.3	CD	AZA 2.2	50	210 ²	176	-16%	Rapid
3	17.8	CD	AZA 1.6	50	276 ²	101	-64%	Rapid
4	14.7	CD	6MP 1.0	50	288 ²	142 ²	-51%	Rapid
5	10.4	UC	6MP 0.6	50	310 ²	206 ²	-34%	Rapid
6	14.5	UC	6MP 0.5	50	330 ²	188 ²	-43%	Rapid
7	11.9	UC	6MP 1.0	50	228	243	+7%	Rapid
8	17.4	CD	AZA 2.2	50	217 ²	221	+2%	Slow
9	6.3	UC	AZA 2.3	50	375	140	-63%	Slow
10	17.5	UC	6MP 1.0	50	647 ²	401 ²	-38%	Slow
11	17.3	UC	6MP 1.0	50	264	278	+5%	Slow
12	16.6	CD	6MP 0.5	50	501	268 ²	-47%	Slow

¹Pmol/8 × 10⁸ erythrocytes; ²This value is the average of two measurements. 5-ASA: 5-aminosalicylate; TGN: 6-thioguanine nucleotides; NAT1: N-acetyltransferase 1; CD: Crohn's disease; UC: Ulcerative colitis; 6MP: 6-mercaptopurine; AZA: Azathioprine.

patients were treated with 6MP (median dosage 1.0 mg/kg, range: 0.5-1.0, equivalent to a median AZA dose of 2.08 mg/kg, range: 1.04-2.08) and 5 with AZA (median dosage 2.2 mg/kg, range: 1.6-2.6). All patients were co-treated with 5-ASA for at least three months at standard doses (50 mg/kg). A total of 36 samples of peripheral blood were collected to measure azathioprine metabolites; on average, 3 samples for patient were collected (range: 2-4). For 8 patients it was not possible to collect two samples before 5-ASA interruption and 2 samples after 5-ASA interruption, because of clinical reasons: therefore 12 samples (5 before and 7 after) were missing; however each patient had at least one sample before and one after 5-ASA interruption. Among these, 19 were obtained during treatment with the thiopurines and 5-ASA, before 5-ASA interruption, and 17 after interruption of 5-ASA and therefore during treatment with the thiopurine alone. The reason for 5-ASA interruption was clinical, mostly simplification of therapy to increase compliance, which is particularly useful in pediatric patients. Samples were taken from the same patient with an interval of at least one month.

Measurement of azathioprine metabolites

Median TGN concentrations before 5-ASA interruption was 270 pmol/8 × 10⁸ erythrocytes (range: 145-750); after the interruption of the aminosalicylate, a 35% reduction (mean absolute value 109 pmol/8 × 10⁸ erythrocytes) in TGN mean concentrations was observed [median 221 pmol/8 × 10⁸ erythrocytes, range: 96-427, coefficient = -0.18, 95%CI: -0.27-(-0.09), *P* value linear mixed effects model 0.0011]. MMPN concentration were not affected significantly by interruption of the aminosalicylate, with a median value of 1059 pmol/8 × 10⁸ erythrocytes, range 246-17943 before the interruption in comparison to a median of 1071 pmol/8 × 10⁸ erythrocytes, range 209-4531 after the interruption (coefficient = -0.13, 95%CI: -0.29-(-0.03), *P* value linear mixed

effects model 0.14). There was a significant correlation between TGN and MMPN concentrations (coefficient 0.3, 95%CI: 0.15-0.45, linear mixed effect *P* value 0.0007) while the dose of thiopurine did not correlate with TGN and MMPN concentrations in these patients. The dose of thiopurine did not change before and after the interruption of the aminosalicylate (median value of azathioprine dose and range 2.08 mg/kg, range: 1.04-2.6).

Demographic and clinical covariates and azathioprine dose and metabolites

For the demographic (gender and age) and clinical (type of IBD and treatment duration) covariates considered, none showed a fully significant effect on the median TGN or MMPN concentrations in a univariate analysis (Table 2).

Genotyping

All polymorphisms considered were respecting Hardy-Weinberg equilibrium and their distribution is comparable to what has been reported in the literature for patients of Caucasian ethnicity. All patients were wild-type for the most relevant ITPA and TPMT variants. For NAT1 genotyping, 7 presented an allele combination corresponding to fast enzymatic activity and 5 to slow activity. NAT2 genotyping revealed that 6 patients presented a genotype corresponding to fast enzymatic activity and 6 to slow activity.

Genotypes and thiopurine metabolites

NAT1 genotypes corresponding to fast enzymatic activity was associated with reduced TGN concentration [coefficient = -0.159, 95%CI: -0.28-(-0.04), *P* value linear mixed effects model 0.033]: mean values were 269.3 and 400.8 respectively in patients with fast and slow NAT1 status before 5-ASA interruption, and 181.9 and 261.6 after 5-ASA interruption. Unexpectedly, the effect of NAT1 status on TGN seems to be persistent even one month since the interruption

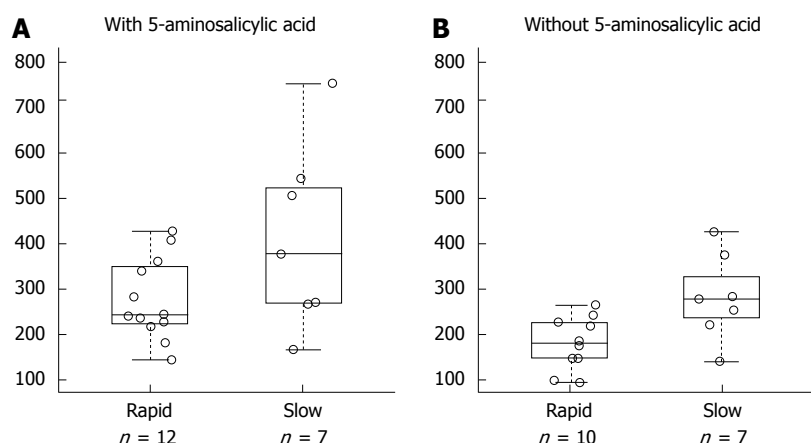


Figure 1 6-thioguanine nucleotides concentration and N-acetyl transferase 1 acetylator status during co-treatment of azathioprine with 5-aminosalicylic acid and after the interruption of the aminosalicylate. A: With 5-aminosalicylic acid; B: Without 5-aminosalicylic acid. A total of 36 samples of peripheral blood were collected from 12 patients to measure azathioprine metabolites; on average, 3 samples for patient were collected (range: 2-4). Among these, 19 were obtained during treatment with the thiopurines and 5-aminosalicylic acid (panel A) and 17 during treatment with the thiopurine alone (panel B). Samples were taken from the same patient with an interval of at least one month. 6-thioguanine nucleotides concentration is expressed as pmol/8 × 10⁸ erythrocytes.

of the aminosalicylate (Figure 1). No effect of NAT1 genotypes was shown on MMPN concentrations. NAT2 genotypes were not related to thiopurine metabolites' concentration in this study.

DISCUSSION

The clinical use of thiopurines in IBDs has increased substantially in recent years; these drugs have indeed a steroid sparing effect^[25] and their use in combination with infliximab has been also advocated^[26]. Coprescription of 5-ASA is also common, and up to 60% of patients on thiopurines are also treated with an aminosalicylate.

Thiopurines are generally well tolerated, however, 15%-20% of patients develop side effects such as leukopenia, hepatitis and pancreatitis^[27-30]. Two key enzymes, TPMT and ITPA, are important for 6MP metabolism: TPMT catalyzes the S-methylation to 6MP and genetic polymorphisms in the TPMT gene are associated with a reduced enzymatic activity and an increased production of the active TGNs; indeed, patients with the homozygous mutation are at high risk of severe and sometimes fatal immunosuppression. For this reason TPMT genotyping or phenotyping is recommended prior to the initiation of therapy. Another important enzyme in thiopurines' metabolism is ITPA; a polymorphism in this gene leads to accumulation of the metabolite 6-thioinosine triphosphate and has been associated with an increased risk of toxicity, in particular pancreatitis, flu like symptoms, rash and gastrointestinal toxicity^[31]; this observation was however not confirmed by other studies^[32,33]. All patients included in our study had a normal TPMT genotype and were wild-type for the most common mutation of ITPA, hence excluding bias due to the influence of these genotypes.

In IBD patients treated with thiopurines an additional

risk results from the co-administration of other drugs, such as the aminosalicylates. In the present study we confirm previous observations^[2,8,9,11,13,34] of a significant decrease in TGN levels after discontinuation of 5-ASA. Furthermore, a dose dependent effect was previously reported for two different 5-ASA doses on thiopurine metabolites levels^[35]. In our study all patients were treated with a dose of 5-ASA of 50 mg/kg, equivalent to the higher dose reported by de Graaf *et al.*^[35]. Consistently with this study, after interruption of 5-ASA we observed an effect on TGN and not on MMPN concentration. This may be due to the different populations considered: TPMT activity indeed is significantly higher in wild-type children (0.08-17 years) than in wild-type adults (aged 18-68 years)^[36].

The mechanism of this interaction is however still unclear. It has been demonstrated that the aminosalicylates inhibit the activity of recombinant TPMT *in vitro*^[37,38], with IC₅₀ values of 78 and 1240 μmol/L for sulfasalazine and 5-ASA respectively. In *in vivo* studies, an increase in TGN levels and in the prevalence of leukopenia was observed in patients treated with azathioprine and 5-ASA; however, short term investigations in patients with IBD^[8-11] did not demonstrate any significant change in TPMT activity. A long term study in patients treated for one year with a high dose (4 g/d) of 5-ASA again did not show any *in vivo* effect on TPMT activity^[39]. It can therefore be concluded that the interaction between aminosalicylates and thiopurines seems not based on inhibition of TPMT, and other pharmacokinetic and/or pharmacodynamic aspects have to be investigated.

5-ASA is orally administered, is poorly absorbed by the gastrointestinal tract and is in part inactivated in the colonic mucosa by NATs^[17]. These enzymes are widely distributed in tissues^[40] and among species^[41], and have important physiological functions; they are also responsible for the N-acetylation of a number

of xenobiotics and drugs including the aminosaliclates. The activity of NAT1 and NAT2 is genetically determined; both genes are located on chromosome 8p22 and a number of polymorphisms have been reported, allowing subjects to be classified as rapid or slow acetylators. The isozymes NAT1 and NAT2 have distinct substrate specificity and the NAT1 isozyme is more important (19000-fold more active) than NAT2 in 5-ASA acetylation *in vitro*^[42]. Interestingly, in our study, patients with the NAT1 slow metabolizer phenotype had significantly higher TGN levels in comparison with rapid metabolizers. The inheritance of a slow acetylator genotype for NAT1 could therefore lead to a reduced inactivation of 5-ASA, and hence to higher blood levels of the aminosaliclate. This could result in a reduction of 6MP inactivation, *via* a still unclear mechanism, with consequent increase in TGN levels. Quite unexpectedly however, this difference was maintained when measurements were performed one month after 5-ASA discontinuation. This may be due to the long half-life of TGN and the fact that a longer period is needed to overcome the reduction in TGN concentrations determined by the increased metabolism of 5-ASA. It is however possible that NAT1 influences TGN concentrations by a different mechanism, not involving 5-ASA metabolism.

As expected, no effect of the NAT2 polymorphism was observed in these patients.

In conclusion, co-administration of 5-ASA and thiopurines is common and probably this association will continue to be prescribed in light of the demonstrated chemopreventive activity for IBD associated colorectal cancer^[7].

Since the number of patients enrolled in this study is limited, this has to be considered a pilot study and more research should be performed to evaluate if the difference in TGN levels observed in patients with the NAT1 slow acetylator phenotype are also related to an increased incidence of thiopurine induced side effects. If this were true, it might be useful to assess the NAT1 genotype before starting therapy and, in those patients with a slow acetylator genotype, it might be prudent to start therapy with a reduced dose of AZA. Moreover, further studies should be performed to evaluate a dose dependent effect of 5-ASA or thiopurine dose on the association between NAT1 status and the pharmacokinetic interaction between 5-ASA and thiopurines: indeed patients with adverse NAT1 status may be treated with low doses of aminosaliclates, maintaining their chemopreventive effect. The effect of NAT1 acetylator status on TGN concentration in patients treated only with thiopurine could also be investigated.

NAT1 genotyping, in addition to careful clinical monitoring and evaluation of thiopurine metabolites, might be a useful guide in those patients receiving azathioprine and aminosaliclates.

COMMENTS

Background

Thiopurines and aminosaliclates are the two most widely used drugs in inflammatory bowel disease (IBD) and are often used in combination. A significant pharmacokinetic interaction has been described for these medications, since this association increases the concentration of thiopurines' active metabolites (TGN).

Research frontiers

Treatment of IBD with thiopurines and aminosaliclates displays significant inter-patient variability in terms of efficacy and incidence of adverse events. Identification of determinants to predict effects of these treatments, such as genetic polymorphisms of enzymes involved in thiopurines and aminosaliclates biotransformation, is of clinical interest.

Innovations and breakthroughs

This article confirms that after interruption of aminosaliclate, concentration of TGNs decrease significantly. Moreover, N-acetyl-transferase 1 (NAT1) acetylator status, relevant for aminosaliclates biotransformation, influences TGN concentration during co-treatment and after the interruption of the aminosaliclate.

Applications

If supported by further clinical studies, NAT1 may be incorporated in multilocus signatures of genotypes useful to predict the efficacy and safety of thiopurine and aminosaliclate co-treatment in young patients with IBD.

Terminology

TGN, the active metabolites of thiopurines, formed after biotransformation of mercaptopurine by enzymes of nucleotides salvage pathway. NAT1, an enzyme that catalyzes the acetylation of amino groups of aminosaliclates such as 5-ASA.

Peer-review

This is an interesting and clinically relevant paper. Obviously the study has a small sample size and thus it is best described as a pilot study. The results of this small study are of interest in that there seems to be some pharmacokinetic interaction between 5-aminosalicylate and thiopurines, there also seems to be a potentially important and previously unexplored effect of NAT polymorphisms.

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Retrospective Study

Computed tomography-guided percutaneous core needle biopsy in pancreatic tumor diagnosis

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Abstract

AIM: To evaluate the techniques, results, and complications related to computed tomography (CT)-guided percutaneous core needle biopsies of solid pancreatic lesions.

METHODS: CT-guided percutaneous biopsies of solid pancreatic lesions performed at a cancer reference center between January 2012 and September 2013 were retrospectively analyzed. Biopsy material was collected with a 16-20 G Tru-Core needle (10-15 cm; Angiotech, Vancouver, CA) using a coaxial system and automatic biopsy gun. When direct access to the lesion was not possible, indirect (transgastric or transhepatic) access or hydrodissection and/or pneumodissection maneuvers were used. Characteristics of the patients, lesions, procedures, and histologic results were recorded using a standardized form.

RESULTS: A total of 103 procedures included in the study were performed on patients with a mean age of 64.8 year (range: 39-94 year). The mean size of the pancreatic lesions was 45.5 mm (range: 15-195 mm). Most (75/103, 72.8%) procedures were performed *via* direct access, though hydrodissection and/or pneumodissection were used in 22.2% (23/103) of cases and indirect transhepatic or transgastric access was used in 4.8% (5/103) of cases. Histologic analysis was performed on all biopsies, and diagnoses were conclusive in 98.1% (101/103) of cases, confirming

3.9% (4/103) of tumors were benign and 94.2% (97/103) were malignant; results were atypical in 1.9% (2/103) of cases, requiring a repeat biopsy to diagnose a neuroendocrine tumor, and surgical resection to confirm a primary adenocarcinoma. Only mild/moderate complications were observed in 9/103 patients (8.7%), and they were more commonly associated with biopsies of lesions located in the head/uncinate process ($n = 8$), than of those located in the body/tail ($n = 1$) of the pancreas, but this difference was not significant.

CONCLUSION: CT-guided biopsy of a pancreatic lesion is a safe procedure with a high success rate, and is an excellent option for minimally invasive diagnosis.

Key words: Computed tomography; Image-guided biopsy; Large-core needle biopsy; Needle biopsy; Pancreatic neoplasms

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Core tip: Histopathologic analysis is often necessary to confirm the diagnosis of pancreatic tumors and aid in treatment planning. Various techniques, such as imaging-guided percutaneous, endoscopic, and surgical biopsies, can be used to obtain material for the cytologic or histologic analysis. In the present study, computed tomography-guided percutaneous core needle biopsies of pancreatic lesions were associated with few complications and 98.1% diagnostic accuracy. The safety and high diagnostic success rate renders this method an excellent minimally invasive option for diagnostic confirmation of solid pancreatic lesions.

Tyng CJ, Almeida MFA, Barbosa PNV, Bitencourt AGV, Berg JAAG, Maciel MS, Coimbra FJF, Schiavon LHO, Begnami MD, Guimarães MD, Zurstrassen CE, Chojniak R. Computed tomography-guided percutaneous core needle biopsy in pancreatic tumor diagnosis. *World J Gastroenterol* 2015; 21(12): 3579-3586 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3579.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3579>

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer deaths worldwide. The most common histologic type is adenocarcinoma, which is extremely aggressive and typically presents as unresectable disease at the time of diagnosis, with an average survival time of six months^[1,2]. For this reason, solid pancreatic masses should be actively investigated to exclude or confirm a diagnosis of pancreatic cancer. The increasing availability of advanced imaging examinations facilitates the diagnosis and staging of pancreatic masses^[2]. However, such methods do not always allow for a precise diagnosis, and noncharacteristic findings can

make it difficult to differentiate adenocarcinoma from other causes of focal pancreatic lesions or benign inflammatory conditions. In such cases, histopathologic analysis can confirm the diagnosis and aid treatment planning. Moreover, diagnosis of adenocarcinoma based on imaging results often still requires preoperative histologic confirmation, as a definitive diagnosis for local unresectable or metastatic tumors is essential for the planning of palliative therapy. Other indications for biopsy include locally advanced pancreatic neoplasia (borderline tumor) requiring neoadjuvant therapy, areas suspected of containing lesions for which treatment is primarily nonsurgical (e.g., focal pancreatitis, tuberculosis, lymphoma, metastases), and patients with high surgical risk^[3].

Various techniques, such as imaging-guided percutaneous, endoscopic, and surgical biopsies, can be used to obtain material for the cytologic or histologic analysis of these lesions^[4]. Surgical biopsies conducted under direct laparotomic or laparoscopic visualization are associated with greater morbidity, mortality, cost, and inpatient time, than are minimally invasive methods. Fine-needle aspiration (FNA) and percutaneous core-needle biopsy guided by endoscopy, ultrasound, or computed tomography (CT), are useful methods for obtaining tissue samples^[4,5]. Although core needle biopsies of pancreatic lesions have been performed since the 1980s, very few studies have evaluated their performance^[6,7]. The objective of this study was to evaluate the techniques, results, and complications related to CT-guided percutaneous core-needle biopsies of solid pancreatic lesions.

MATERIALS AND METHODS

Study design

This was a retrospective study analyzing CT-guided percutaneous biopsies of solid pancreatic lesions performed at a cancer reference center between January 2012 and September 2013. The study was approved by the Institutional Ethics Review Board. All study participants or their legal guardians provided informed written consent prior to the procedure.

Biopsy procedure

An interventional radiologist or a resident doctor under supervision performed the procedures. Routine coagulation tests were collected and evaluated before the procedures. Each case was individually assessed to determine the best access route, with direct access preferred whenever possible. All procedures were performed under local anesthesia with 2% lidocaine; conscious sedation was used in some cases to increase the patients' comfort. Depending on the distance between the lesion and the skin, a 16-20 G Tru-Core needle (10-15 cm; Angiotech, Vancouver, CA) was used with a coaxial system and automatic biopsy gun to obtain biopsy specimens. When necessary, iodinated contrast was administered intravenously to better

characterize the target lesion and adjacent vascular structures.

When abdominal structures prevented direct access to the lesion, indirect (transgastric or transhepatic) access or hydrodissection and/or pneumodissection maneuvers were used. For these procedures, a coaxial needle was used to inject 0.9% saline (hydrodissection) or 50-100 mL ambient air (pneumodissection) to displace adjacent structures from the needle's trajectory. The choice between these techniques depended on the position in which the procedure was performed and the structures involved, as injected air tends to concentrate in the uppermost areas of the abdomen.

CT examinations were performed immediately after all procedures to identify any bleeding and/or other complications. Patients remained under observation for ≥ 1 h after the procedure; asymptomatic patients with stable vital signs were released upon receiving guidance concerning later complications. An additional CT was performed for patients exhibiting symptoms or changes in vital signs to rule out further complications.

Data collection

A standardized form was used to collect patient (age, sex, presence of primary neoplasia), lesion (location in the pancreas, lesion dimensions, and resectability based on imaging), and procedure (indication, needle caliber, access, immediate and subsequent complications) characteristics, as well as histologic results from the biopsy (sufficiency of biopsy material, benign or malignant status, tumor type, and associated surgical findings).

Statistical analysis

All data were analyzed using SPSS software (version 20.0; IBM Corp., Armonk, NY, United States). Descriptive analysis was performed to calculate simple and relative frequencies of the study variables. Dichotomous scalar variables were compared using Student's *t* or nonparametric Mann-Whitney *U* tests. Analysis of variance or the nonparametric Kruskal-Wallis test was used for comparison of variables with three or more possible values. Categorical variables were examined using 2×2 and 2×3 tables and Pearson's χ^2 test with Yates correction or Fisher's exact test, as needed, to evaluate statistical significance. $P \leq 0.05$ was considered as statistically significant. The statistical methods used in this study were reviewed by a biomedical statistician from the AC Camargo Cancer Center.

RESULTS

A total of 103 procedures in patients with a mean age of 64.8 y (range: 39-94 year) were included. Of these, 50.5% (52/103) were women, 20.4% (21/103) had histories of previous primary tumors, most commonly lung cancer ($n = 4$).



Figure 1 Percutaneous computed tomography-guided core needle biopsy of a pancreatic lesion using direct access. Non-enhanced axial computed tomography of the upper abdomen showing an anteriorly inserted coaxial needle (17 G) that is placed directly over the lesion located at the head of the pancreas (arrow).

The majority (65/103, 63.1%) of lesions was located in the head/uncinate process, 27.2% (28/103) were in the body, and 9.7% (10/103) were in the tail of the pancreas. The mean size of the pancreatic lesions was 45.5 mm (range: 15-195 mm). Based on imaging criteria, 77.7% (80/103) of lesions were unresectable; 22 (21.4%) were resectable and one (1.0%) was a borderline case. Highly suspected pancreatic masses were found in 6.8% (7/103) of patients ($n = 6$ located in the head and 1 in the body/tail), for which echoendoscopy-guided FNAs were performed before the biopsies, with negative results.

Most (75/103; 72.8%) procedures were performed *via* direct access (Figure 1). The remaining cases were performed with hydrodissection ($n = 16$), pneumodissection ($n = 5$), or hydro/pneumodissection ($n = 2$) maneuvers, or with indirect transhepatic ($n = 3$) or transgastric ($n = 2$) access (Figures 2, 3, 4 and 5). Access was anterior in 77.7% (80/103) of cases, posterior or paravertebral in 14.6% (15/103), and left lateral in 7.8% (8/103) of cases. An 18 G needle was used in most (100/103; 97.1%) cases; 20 G ($n = 2$) and 16 G ($n = 1$) needles were used in the remaining cases. The mean length of the coaxial needle's trajectory from the skin to the lesion was 84.3 mm (range: 9-158 mm).

Histologic analyses were performed on all biopsies (Figures 6 and 7). Diagnoses were conclusive in 98.1% (101/103) of cases, confirming 3.9% (4/103) of tumors as benign and 94.2% (97/103) as malignant: 84.5% (87/103) were adenocarcinomas, 2.9% (3/103) were neuroendocrine tumors, 5.8% (6/103) were metastases, and 1.0% (1/103) was a primary leiomyosarcoma. Of the patients with tumors metastasizing to the pancreas, two had pulmonary adenocarcinoma and one each had esophageal cancer, choroidal melanoma, a neuroendocrine tumor, and a solitary fibrous tumor. Only two cases had inconclusive biopsy results, requiring a second biopsy to diagnose

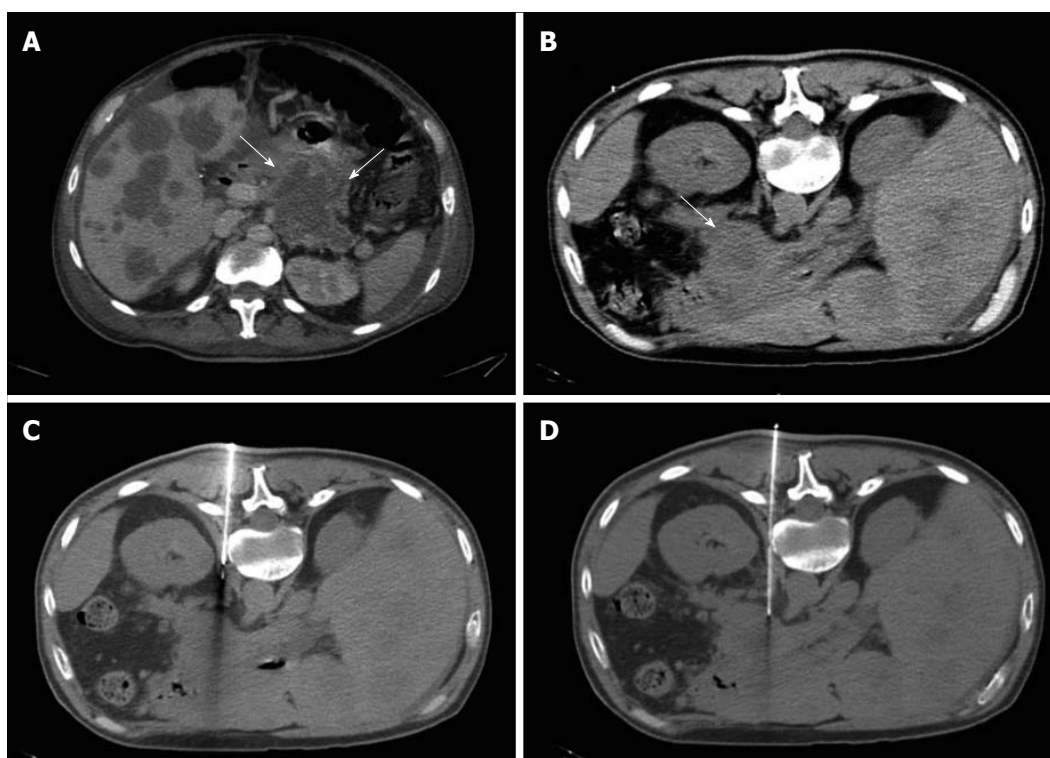


Figure 2 Percutaneous computed tomography -guided core needle biopsy of a pancreatic lesion using hydrodissection. A: Contrast-enhanced computed tomography (CT) showing an expansive lesion in the tail of the pancreas (arrows). An anterior approach was considered difficult because of the interposition of the intestine in the supine position, therefore, a posterior approach was used; B: Non-enhanced axial CT of the upper abdomen in the prone position showing a lesion located in the tail of the pancreas (arrow); C: Coaxial needle (17 G) positioned in the pillar of the diaphragm, where saline solution was injected to enlarge the paravertebral space, enabling an unobstructed needle path; D: Biopsy needle (18 G) placed adjacent to the pancreatic lesion.

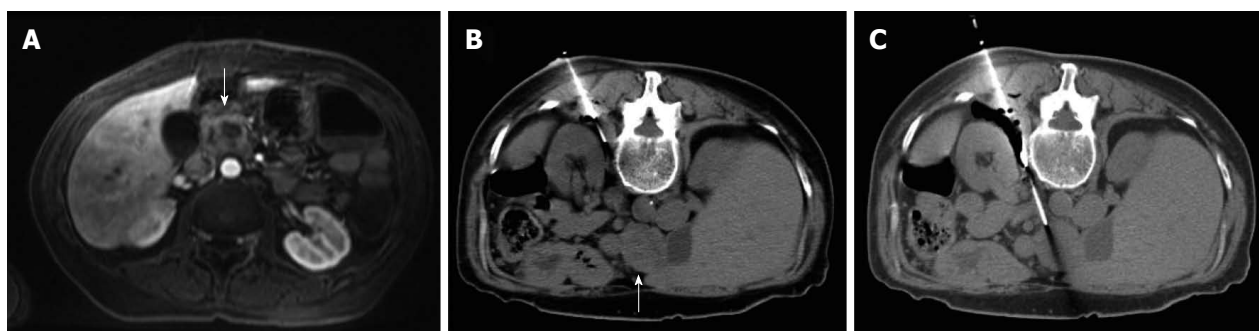


Figure 3 Percutaneous computed tomography-guided core needle biopsy of a pancreatic lesion using pneumodissection. A: Contrast-enhanced magnetic resonance axial image showing a heterogeneous lesion in the body of the pancreas (arrow). The anterior approach was considered difficult because of the interposition of the intestine in the supine position, therefore, a posterior approach was used; B: Non-enhanced axial computed tomography in the prone position showing the pancreatic lesion (arrow) and the coaxial needle (17 G) positioned in the left pararenal space, where air was injected to displace the kidney and adjacent vessels; C: Biopsy needle (18 G) placed adjacent to the pancreatic lesion.

a neuroendocrine tumor, and surgical resection to confirm a primary adenocarcinoma. CT-guided biopsy confirmed adenocarcinoma in the seven patients who had negative echoendoscopy-guided FNA results.

No major procedure-related complications were observed. Mild to moderate complications occurred in 8.7% (9/103) of patients, which resolved spontaneously without treatment; retroperitoneal and subcapsular hepatic hematomas ($n = 6$) occurred immediately after biopsy, and bleeding, symptomatic, and asymptomatic pancreatitis ($n = 3$) developed later. Patients with complications did not

differ from those without complications with respect to age, lesion size, or lesion-skin distance (Table 1). Complications were more commonly associated with biopsies of lesions located in the head/uncinate process, and did not differ according to biopsy technique, access type, or needle caliber (Table 2). There were no suspicious cases of tumor seeding in this case series.

DISCUSSION

CT-guided core needle biopsy is a well-established

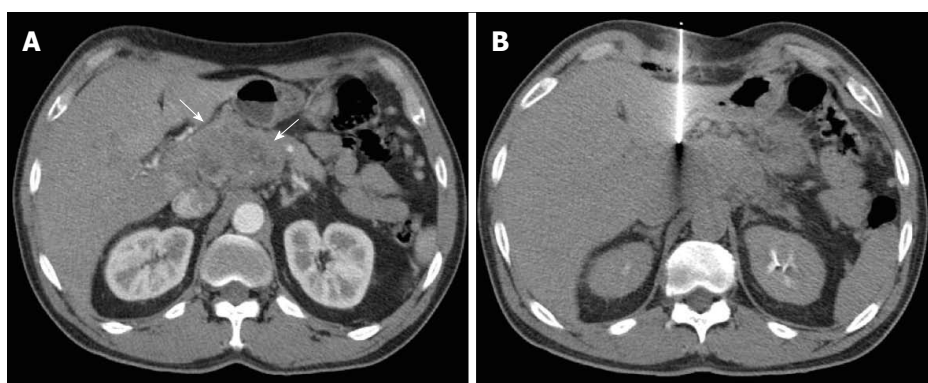


Figure 4 Percutaneous computed tomography-guided core needle biopsy of a pancreatic lesion using transhepatic access. A: Contrast-enhanced computed tomography showing an expansive lesion in the head and body of the pancreas (arrows). As the patient could not stay in the prone position, the posterior approach was not possible. Therefore, an anterior transhepatic approach was used; B: Biopsy needle (18 G) tip placed adjacent to the pancreatic lesion through the left liver lobe.

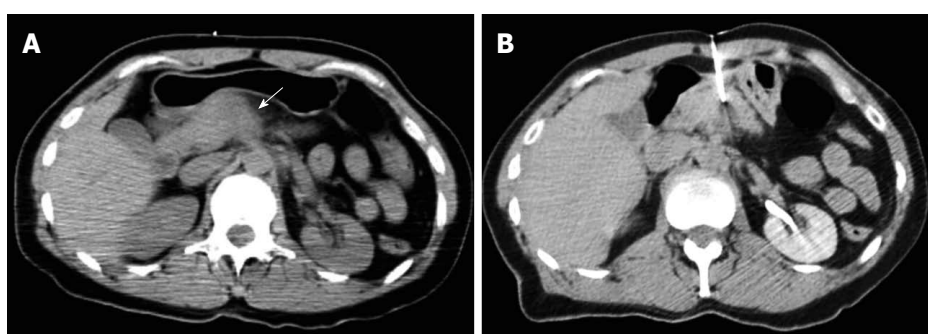


Figure 5 Percutaneous computed tomography-guided core needle biopsy of a pancreatic lesion using transgastric access. A: Non-enhanced computed tomography showing a poorly defined nodule in the body of the pancreas (arrow). A posterior approach was considered difficult because of the interposition of large vessels in the needle path in the prone position, therefore, an anterior transgastric approach was used; B: Biopsy needle (20 G) placed adjacent to the pancreatic lesion through the stomach.



Figure 6 Biopsied specimens of a percutaneous computed tomography-guided core-needle biopsy of a pancreatic lesion.

routine procedure for obtaining samples from various organs^[8-10]. Most studies, including this one, demonstrate that this procedure has > 90% diagnostic accuracy for pancreatic lesions (Table 3)^[2,4,5,11-13]. In our cohort, histologic diagnosis based on percutaneous biopsy samples was considered indeterminate in only two cases, which were later confirmed to be malignant.

Core needle biopsy is considerably more sensitive

Table 1 Patient and lesion characteristics for biopsies with and without complications

Variable	Without complications (<i>n</i> = 94)	With complications (<i>n</i> = 9)	<i>P</i> value
Patient age, yr	64.9 ± 12.9	64.1 ± 12.3	0.86
Lesion size, mm	46.4 ± 25.9	35.5 ± 14.2	0.25
Lesion-skin distance, mm	83.1 ± 31.5	97.1 ± 30.2	0.20

Table 2 Incidence of complications after computed tomography-guided biopsy of pancreatic lesions *n* (%)

Variable	Complications	<i>P</i> value
Lesion location		
Head/uncinate process	8 (12.3)	0.15
Body/tail	1 (2.6)	
Biopsy technique		
Direct	4 (5.4)	0.11
Indirect	5 (17.2)	
Access type		
Anterior	8 (10.0)	0.60
Posterior/paravertebral	1 (6.7)	
Needle caliber		
18 G	9 (9.0)	0.86

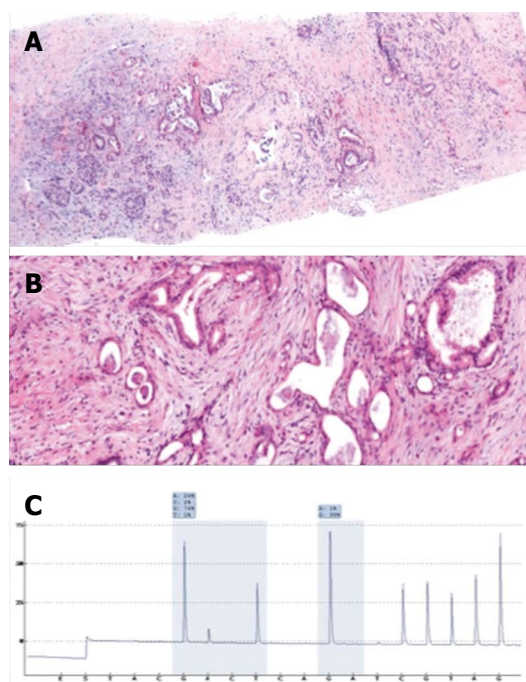


Figure 7 Pathologic and molecular analysis of a biopsied specimen. A: Panoramic view ($\times 10$) and B: High-power field ($\times 40$) of a pancreatic core biopsy with hematoxylin and eosin staining showing atypical and irregularly displayed ductal structures in a desmoplastic stroma compatible with the diagnosis of ductal adenocarcinoma of the pancreas; C: Pyrogram demonstrating a mutation in codon 12.

than FNA for the diagnosis of pancreatic diseases, particularly when the differential diagnosis includes neuroendocrine tumors and chronic pancreatitis^[14,15]. The major advantage is sample quality, as the core needle extracts sufficient material to determine cell type and origin *via* histologic and immunohistochemical analyses, thus allowing reliable differentiation of pancreatic tumor types^[2,11]. In addition, core needle biopsy does not require that a cytopathologist be present during the procedure.

Ultrasound can be used to guide percutaneous core needle and FNA biopsies of pancreatic lesions, with the advantages of lower cost, shorter procedure time, elimination of ionizing radiation, and the ability to accompany the needle's trajectory in real time^[14,16]. However, accessing small lesions and those located in the body/tail of the pancreas is difficult with ultrasound guidance, particularly in the presence of gaseous gastrointestinal interpositioning. The choice of imaging method to guide the procedure should be individualized and depend on the interventional radiologist's experience and the patient and lesion characteristics.

As demonstrated in the present study, percutaneous biopsy typically involves anterior access to pancreatic lesions. However, pancreatic masses in the head or uncinate process are frequently obscured by other abdominal structures, including the stomach, duodenum, transverse colon, liver, mesenteric vessels, and/or inferior vena cava. In such cases, indirect (*e.g.*,

Table 3 Accuracy and complications of image-guided percutaneous needle biopsy of pancreatic lesions

Ref.	n	Imaging method	Accuracy	Complications
Brandt <i>et al</i> ^[15]	269	US and CT	93%	1.10%
Karlson <i>et al</i> ^[11]	100	US	89%	-
Paulsen <i>et al</i> ^[5]	107	US and CT	94.40%	2.80%
Amin <i>et al</i> ^[2]	372	US and CT	90%	4.60%
Yu <i>et al</i> ^[12]	43	CT	94.30%	2.30%
Yang <i>et al</i> ^[13]	88	US	93%	-
Current study	103	CT	98.10%	8.70%

CT: Computed tomography; US: Ultrasound.

transhepatic, transgastric, or transcaval) access is considered a safe alternative, though accompanied by risks of bleeding and peritonitis^[17,18]. Another alternative includes the hydrodissection and pneumodissection maneuvers^[19,20]. The location of the lesion can be confirmed with intravenous contrast administration to improve the diagnostic accuracy of the procedure^[21].

Previous reports indicate that serious complications related to this procedure are rare, even when biopsies are conducted *via* indirect access^[2,5,11-15,17,18,21]. Accordingly, no serious complications occurred in the patients in our study, with only a small percentage of patients experiencing mild adverse events that spontaneously resolved. However, Amin *et al*^[2] reported complications in 17/372 (4.6%) percutaneous core needle biopsies, including serious complications such as abscess, duodenal perforation, and large retroperitoneal hematoma. Almost all of the complications in the present study occurred from lesions located in the head/uncinate process of the pancreas, possibly resulting from the large number of vessels and structures involving this portion of the organ. Needle size did not relate to the complication rate, though most procedures were performed with the same size needle (18 G).

Although rare, the peritoneal implantation of tumor cells during biopsy is a complication of concern. Cases of tumor dissemination along the needle trajectory after percutaneous or echoendoscopy-guided FNA have been described^[22-24]. This did not occur in any of our cases. Some authors have suggested that the use of a coaxial needle, as used in this study, reduces the risk of implantation along the biopsy trajectory as the adjacent normal tissues are shielded from the needle^[25].

Echoendoscopy-guided FNA has received attention because of its accuracy in obtaining cytopathologic material, with a sensitivity of 55%-97%^[26-28]. This technique facilitates access to small lesions near the duodenum and stomach. However, despite the increasing availability of this technique, success rates with this method are difficult to reproduce, with high rates of false-negative findings^[29]. In comparison, CT-guided biopsy has a greater availability, lower cost, higher success rate, and allows access to lesions in any part of the pancreas. Notably, pancreatic

adenocarcinoma was confirmed by CT-guided biopsy in seven patients in our sample who had negative echoendoscopy-guided FNA results. However, those results were obtained from FNA procedures performed at outside centers and were not properly reviewed.

This study has several limitations. Given its retrospective nature, we could not standardize medium- and long-term follow-up evaluations. Moreover, our sample only included patients attending a single cancer reference center that were selected to undergo biopsy based on discussions among the oncologist, surgeon, and interventional radiologist. Furthermore, we were unable to directly compare CT-guided core needle biopsy with echoendoscopy-guided FNA, as this method was not available in our institution during the study period. Future studies are needed to address this issue.

In the present sample, CT-guided percutaneous core needle biopsies of pancreatic lesions were associated with few complications and a 98.1% diagnostic accuracy. The results demonstrate that this procedure is safe when performed by an experienced interventional radiologist, and represents a valuable alternative for preoperative diagnostic confirmation of solid pancreatic lesions.

COMMENTS

Background

Pancreatic cancer is the fourth leading cause of cancer deaths worldwide. Histopathologic analysis is often necessary for diagnosis confirmation and treatment planning, for which samples can be obtained using various techniques, such as imaging-guided percutaneous, endoscopic, and surgical biopsies. This study evaluates the techniques, results and complications related to computed tomography (CT)-guided percutaneous core needle biopsies of solid pancreatic lesions.

Research frontiers

CT-guided core needle biopsy is a well-established procedure used routinely to obtain samples from various organs. Compared to surgical biopsies, this method is associated with lower morbidity, mortality, cost, and inpatient time.

Innovations and breakthroughs

Although core needle biopsies of pancreatic lesions have been performed since the 1980s, very few studies have evaluated their performance. This work is the first to describe different techniques for CT-guided percutaneous biopsy of pancreatic masses, demonstrating a higher diagnostic accuracy than previously reported.

Applications

Core needle biopsy is considerably more sensitive than fine-needle aspiration for the diagnosis of pancreatic diseases, particularly when the differential diagnosis includes neuroendocrine tumors and chronic pancreatitis. The major advantage of this technique is sample quality, as the core needle extracts sufficient material to determine cell type and origin *via* histologic and immunohistochemical analyses, thus allowing reliable differentiation of pancreatic tumor types.

Terminology

Core needle biopsy involves the use of a long metal needle to collect tissue cores from a lesion. This procedure is safe when guided by imaging methods and performed by an experienced interventional radiologist.

Peer-review

The authors described the usefulness of CT-guided needle biopsy for diagnosing pancreatic tumors. The article is well written with sufficient sample size and acceptable procedure/results. This procedure appears safe and should be widely used for clinical diagnosis of pancreatic cancer. In general, the method is

valuable for confirming pancreatic cancer along with pathologic testing.

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Retrospective Study

Appropriate empirical antibiotic use and 30-d mortality in cirrhotic patients with bacteremia

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Ethics approval: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008, and was reviewed and approved by the Institutional Review Board at Sanggye Paik Hospital.

Informed consent: Waived by the Institutional Review Board.

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Abstract

AIM: To analyze whether prompt and appropriate empirical antibiotic (AEA) use is associated with mortality in cirrhotic patients with bacteremia.

METHODS: A total of 102 episodes of bacteremia in 72 patients with cirrhosis were analyzed. AEA was defined as a using or starting an antibiotic appropriate to the isolated pathogen at the time of bacteremia. The primary endpoint was 30-d mortality.

RESULTS: The mortality rate at 30 d was 30.4% (31/102 episodes). Use of AEA was associated with better survival at 30 d (76.5% vs 46.9%, $P = 0.05$), and inappropriate empirical antibiotic (IEA) use was an independent factor associated with increased mortality (OR = 3.24; 95%CI: 1.50-7.00; $P = 0.003$, adjusted for age, sex, Child-Pugh Class, gastrointestinal bleeding, presence of septic shock). IEA use was more frequent when the isolated pathogen was a multiresistant pathogen, and when infection was healthcare-related or hospital-acquired.

CONCLUSION: AEA use was associated with increased survival of cirrhotic patients who developed bacteremia. Strategies for AEA use, tailored according to the local epidemiological patterns, are needed to improve survival of cirrhotic patients with bacteremia.

Key words: Liver cirrhosis; Bacteremia; Appropriate antibiotics; Survival; Multiresistant pathogen

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Core tip: Appropriate empirical antibiotic use was associated with improved survival in cirrhotic patients with bacteremia, indicating the importance of initial antibiotic selection.

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INTRODUCTION

Bacterial infections are very frequent in patients with advanced cirrhosis^[1]. Patients with cirrhosis have altered and impaired immunity, which favors bacterial translocation, and may explain the high incidence of bacterial infection^[2]. Patients with cirrhosis are not only at increased risk of developing bacterial infection^[3], but are also at increased risk of death from bacterial infection compared with individuals without cirrhosis^[4,5]. Bacterial infection is a major cause of death in cirrhosis patients^[6]. Therefore, early diagnosis and treatment of bacterial infection is pivotal in the management of these patients^[6]. In a heterogeneous patient population with septic shock, early initiation of appropriate empirical antibiotic (AEA) therapy was associated with a higher survival rate^[7-9]. However, little data exist on the association between AEA use and outcome in patients with cirrhosis. Therefore, in this study, we assessed whether AEA use is associated with survival in cirrhotic patients who developed bacteremia.

MATERIALS AND METHODS

Study design, setting and participants

A retrospective, historical cohort was identified by reviewing the database of Sanggye Paik Hospital, Inje University School of Medicine, Seoul, South Korea, between January 2008 and December 2011. During the study period, a total of 114 episodes of bacteremia were identified. Cirrhosis was defined clinically, when indicators of cirrhosis were present, including thrombocytopenia (platelet count $< 150 \times 10^3/L$), splenomegaly (by cross-sectional images), ascites (by cross-sectional images or the use of diuretics for control of ascites), varices (by upper endoscopy, cross-sectional images, or a history of variceal bleeding), and a cirrhotic liver in cross-sectional imaging studies (nodular liver surface or caudate lobe hypertrophy)^[10]. We excluded 12 episodes of bacteremia that were

transferred to other hospitals within 30 d. Finally, a total of 102 episodes of bacteremia were included and analyzed. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008, and was approved by the Institutional Review Board at Sanggye Paik Hospital.

Variables and definitions

From the medical records, we collected data on age, sex, height, weight, admission date, causes of cirrhosis, presence of gastrointestinal bleeding, ascites, encephalopathy, discharge date, and mortality. We also collected data on the isolated pathogen, and antibiotics used, as well as systolic blood pressure, diastolic blood pressure, heart rate, body temperature, respiration rate, white blood cell (WBC) count, neutrophil count, platelet count, prothrombin time, bilirubin, albumin, creatinine, sodium, aspartate aminotransferase, alanine aminotransferase, and C-reactive protein on the day of bacteremia. The type of infection was defined as community-acquired, if diagnosed within 48 h of admission without hospitalization in the previous 6 mo; healthcare-associated, if diagnosed within 48 h of admission in patients hospitalized for at least 2 d in the previous 6 mo; and hospital-acquired if diagnosed 48 h after admission^[11].

Infections were defined as follows^[11,12]: (1) spontaneous bacteremia; positive blood cultures without a source of infection; (2) spontaneous bacterial peritonitis (SBP): ascitic fluid polymorphonuclear cells $> 250/\mu L$ with/without a positive fluid culture; (3) lower respiratory tract infections: new pulmonary infiltrate in the presence of: (a) at least one respiratory symptom (cough, sputum production, dyspnea, pleuritic pain) with (b) at least one finding on auscultation (rales or crepitation) or one sign of infection (core body temperature $> 38^\circ C$ or $< 36^\circ C$) in the absence of antibiotics; (4) skin infection: fever with cellulitis; (5) urinary tract infection (UTI): urine WBC > 15 /high-power field with either positive urine Gram stain or culture in a symptomatic patients; and (6) other sources of infection; (e.g., intraabdominal abscess, cholecystitis, secondary peritonitis).

Systemic inflammatory response syndrome (SIRS) was defined when 2 or more of the following criteria were present: (1) a core temperature $\geq 38^\circ C$ or $\leq 36^\circ C$; (2) a heart rate ≥ 90 beats/min; (3) tachypnea ≥ 20 breaths/min or partial carbon monoxide pressure ≤ 32 mmHg or the need of mechanical ventilation; and (4) a WBC count $\geq 12 \times 10^9/L$ or $\leq 4 \times 10^9/L$ or $> 10\%$ of immature neutrophils^[13]. Septic shock was defined as patients with SIRS plus persistent hypotension requiring therapy with vasopressors^[14]. AEA use was defined as an antimicrobial with *in vitro* activity appropriate for the isolated pathogen^[14]. Otherwise, the initial therapy was considered inappro-

Table 1 Isolated bacteria *n* (%)

	<i>n</i> = 102
Gram (+)	
Staphylococcus aureus	13 (13)
Coagulase negative staphylococci	21 (21)
Streptococcus species	7 (7)
Enterococcus species	8 (8)
¹ Other Gram (+) pathogens	3 (3)
Gram (-)	
Escherichia coli	16 (16)
Klebsiella pneumoniae	18 (18)
Pseudomonas aeruginosa	3 (3)
Acinetobacter baumannii	5 (5)
² Other Gram (-) pathogens	8 (8)

¹Other Gram (+) pathogens were *Micrococcus luteus* (*n* = 2), and *Leuconostoc* (*n* = 1); ²Other Gram (-) pathogens were *Aeromonas* (*n* = 3), *Citrobacter* (*n* = 1), *Flavobacterium* (*n* = 1), *Morganella* (*n* = 1), *Serratia* (*n* = 1), *Vibrio vulnificus* (*n* = 1).

appropriate empirical antibiotics (IEA) therapy.

Multiresistant pathogens were defined as the following in the current study: extended-spectrum beta-lactamase-producing bacteria (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) or derepressed chromosomal AmpC beta-lactamase producing *Enterobacteriaceae* (e.g., *Enterobacter* or *Citrobacter* spp.), *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Achromobacter* spp., methicillin-resistant *Staphylococcus aureus*, and *Enterococcus faecium*.

Statistical analysis

The primary endpoint variable was survival at 30 d. The difference in 30-d survival rate was compared between patients who received AEA and those who did not, using the Kaplan-Meier method with the log-rank test. Cox regression analysis was conducted to identify factors associated with 30-d mortality. Multivariable Cox-regression analysis was conducted to determine the independent factors using variables with *P* < 0.05 in the univariable analysis. Age and sex were included in the multivariable model, irrespective of the *P* value in the univariable analysis. A *P*-value < 0.05 was considered significant. The statistical methods in this study were reviewed by Dong Hyun Sinn, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea.

RESULTS

Baseline characteristics

During the study period, 102 episodes of bacteremia in 72 patients (52 male, 20 female; mean age: 57.6 ± 11.0 years) were identified. Thirty patients had multiple episodes (range: 2-4) of bacteremia during the study period. Alcohol was a major cause of cirrhosis (34/72, 47.2%) followed by hepatitis B (25/72, 35.7%), non-B, non-C cirrhosis (8/72, 11.1%)

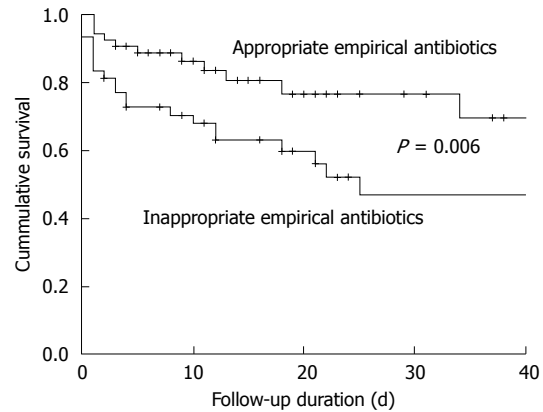


Figure 1 30-d survival rate according to the use of appropriate empirical antibiotics. The 30-d survival rate was significantly higher in patients treated with appropriate empirical antibiotics (81.5% vs 56.2%, *P* = 0.006).

and hepatitis C (5/72, 6.9%). Four patients had hepatocellular carcinoma.

Infection type, site, isolated pathogen

Of the 102 episodes of bacteremia, 23 (22.5%) were community-acquired, 3 (2.9%) were healthcare-associated, and 69 (67.6%) were hospital-acquired. SIRS was noted in 68 episodes (66.7%). Septic shock was present in 27 episodes (26.5%). The most frequent origin of bacteremia was spontaneous bacteremia (33.3%), followed by SBP (30.4%), UTI (16.7%), lower respiratory tract infection (8.8%), soft tissue infection (4.9%), intra-abdominal abscess (3.0%), cholecystitis (2.0%), and secondary peritonitis (1.0%). The isolated pathogens are shown in Table 1.

AEA use and 30-d mortality

The mortality rate at 30-d was 30.4% (31/102 episodes). The 30-day mortality was significantly lower in patients treated with AEA (18.5% vs 43.8%, *P* = 0.006, Figure 1). In the univariable analysis, use of AEA, Child-Pugh class (C vs A/B), gastrointestinal bleeding (yes vs no), and presence of septic shock (yes vs no) were also factors associated with 30-d mortality (Table 2). Age, sex, multiresistant pathogen, infection type (hospital- or healthcare-acquired vs community-acquired), and presence of SIRS were not associated with 30-d mortality. In the multivariable analysis, Child-Pugh Class (C vs A/B), AEA use, gastrointestinal bleeding, and presence of septic shock were independent factors associated with 30-d mortality (Table 2).

AEA use and 30-d mortality in subgroups

In subgroup analysis, the 30-d mortality was significantly higher for no AEA use in patients with Child-Pugh Class C [66.7% (18/27) vs 30.3% (10/33), *P* = 0.005], with SIRS [53.1% (17/32) vs 19.4% (7/36), *P* = 0.004], or with septic shock [85.7% (12/14) vs 30.8% (4/13), *P* = 0.006]. The 30-d

Table 2 Factors associated with 30-d mortality

Factors	Univariate	P value	Multivariate	P value
Age (per years)	1.00 (0.97-1.03)	0.89		
Male (<i>vs</i> female)	0.92 (0.42-2.1)	0.84		
Child-Pugh class (C <i>vs</i> A/B)	7.93 (2.40-26.1)	0.01	7.18 (2.07-24.91)	0.002
Multiresistant pathogen	1.49 (0.73-3.06)	0.26		
Inappropriate antibiotic use	2.00 (0.97-4.13)	0.06	3.24 (1.50-7.00)	0.003
Gastrointestinal bleeding	2.12 (1.01-4.43)	0.05	2.92 (1.33-6.38)	0.007
Infection type (community <i>vs</i> non-community)	0.76 (0.35-1.64)	0.49		
Systemic inflammatory response syndrome	1.79 (0.77-4.16)	0.17		
Septic shock	4.26 (2.10-8.66)	< 0.01	3.39 (1.56-7.37)	0.002

mortality was also higher in Child-Pugh Class A/B [14.3% (3/21) *vs* 0% (0/21), $P = 0.23$], in patients without SIRS [25.0% (4/16) *vs* 16.7% (3/18), $P = 0.68$], and in patients without septic shock [26.5% (9/34) *vs* 14.6% (6/41), $P = 0.25$], although the difference was not statistically significant.

Factors associated with AEA use

IEA use was much higher in hospital-acquired infection (55.1%, 38/69) and healthcare-associated infection (66.7%, 2/3) than in community-acquired infection (26.7%, 8/30, $P = 0.02$). IEA use was also much higher when the isolated organism was a multiresistant pathogen [80.0%, (36/45) *vs* 21.1%, (12/57), $P = 0.01$], but was not different according to infection site, presence of SIRS, septic shock, or Child-Pugh Class.

DISCUSSION

In this study, the 30-d mortality rate in cirrhotic patients with bacteremia was 30.4%. The independent predictors for mortality were liver function (Child-Pugh Class C), gastrointestinal bleeding, presence of septic shock, and IEA use. IEA use was significantly associated with mortality, especially for patients with poor liver function (Child-Pugh class C), with SIRS or with septic shock. The major reason for IEA use was a multiresistant pathogen, usually in the setting of hospital-acquired or healthcare-associated infection.

Patients with liver cirrhosis are known to have impaired immunity and are predisposed to infection^[15]. Furthermore, when infections develop, they are known to increase mortality 4-fold in these patients^[16]. Compared to non-cirrhotic patients, cirrhotic patients showed poor prognosis in community-acquired bacteremia^[17,18], community-acquired pneumonia^[19], and any bacteremia^[5]. In this study, we also observed a high mortality rate in our series (30.4%) when cirrhotic patients developed bacteremia. In this study, advanced liver disease (Child-Pugh Class C) was found to be an independent prognostic factor for mortality, indicating the importance of underlying liver function as a prognostic marker. Consistent with our finding, several studies also showed that fatal outcomes were more frequent in patients with

advanced cirrhosis^[15,17,20]. Certainly, liver function is an important predictor in mortality. However, in addition to liver function, gastrointestinal bleeding, presence of septic shock, and IEA use were independent factors associated with mortality in this study. Of these factors, a modifiable, physician-dependent factor is AEA use. Indeed, several previous studies have shown the importance of AEA use in the setting of septic shock^[7-9,14]. In line with a previous study in cirrhosis patients^[14], we also noted that AEA use was a crucial component in improving the outcome of cirrhotic patients with bacteremia.

Generally, intravenous third generation cephalosporins are recommended as an empirical antibiotic therapy for cirrhotic patients^[6]. However, increased incidences of Gram-positive and drug-resistant organisms have been reported, particularly in hospital-acquired infections and in patients receiving quinolone prophylaxis^[21]. Nosocomial infection is a well-known predictor of multiresistant bacteria, along with long-term norfloxacin prophylaxis, recent infection by multiresistant bacteria, and recent use of beta-lactams^[22]. Hospitalized patients with cirrhosis have the highest risk of developing infections^[23], and secondary infections that develop in a hospital setting are a predictor of mortality in cirrhotic patients^[11]. We also observed that IEA use was mainly due to multiresistant pathogens, mainly in the setting of hospital-acquired or healthcare-related infection.

Although the data are limited by being a retrospective study with a small sample size, they demonstrate the importance of AEA use on the outcome of cirrhotic patients with bacteremia. The impact of AEA on survival of patients with bacteremia was significant, especially those with Child-Pugh Class C, SIRS, or septic shock. IEA use was mainly due to multiresistant pathogens. Strategies for AEA use, tailored according to the local epidemiological patterns, are needed to improve survival of cirrhotic patients with bacteremia.

COMMENTS

Background

Bacterial infections are very frequent in patients with advanced cirrhosis. Patients with cirrhosis are not only at increased risk of developing bacterial

infection, but are also at increased risk of death from bacterial infection compared with individuals without cirrhosis. Currently, little data exist on the association between appropriate empirical antibiotic (AEA) use and outcome in patients with cirrhosis.

Research frontiers

The association between AEA use and survival of cirrhotic patients who developed bacteremia was analyzed.

Innovations and breakthroughs

AEA use was associated with better survival at 30 d and inappropriate empirical antibiotic (IEA) use was an independent factor associated with increased mortality. IEA use was more frequent when the isolated pathogen was multiresistant, and when the infection was healthcare-related or hospital-acquired infection.

Applications

This data suggest that AEA use is an important factor in improving the outcome of cirrhotic patients with bacteremia. Strategies for AEA use, tailored according to the local epidemiological patterns, are needed to improve survival of cirrhotic patients with bacteremia.

Terminology

AEA use was defined if an the antimicrobial had *in vitro* activity appropriate for the isolated pathogen.

Peer-review

Bacterial infections are the major cause of death in cirrhotic patients. This manuscript described the retrospective investigation of the importance of appropriate empirical antibiotics in treatment of cirrhotic patients with bacteremia. Although this study is lack of novelty due to the publications of several other studies on the similar subject, the experiments conducted in this study have rationally been designed and carried out, and the elementary internal connections between use of appropriate empirical antibiotics and outcome in patients with cirrhosis have been revealed in this study.

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Retrospective Study

Formalin irrigation for hemorrhagic chronic radiation proctitis

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Abstract

AIM: To assess the efficacy and safety of a modified topical formalin irrigation method in refractory hemorrhagic chronic radiation proctitis (CRP).

METHODS: Patients with CRP who did not respond to previous medical treatments and presented with grade II-III rectal bleeding according to the Common Terminology Criteria for Adverse Events were enrolled. Patients with anorectal strictures, deep ulcerations, and fistulas were excluded. All patients underwent flexible endoscopic evaluation before treatment. Patient demographics and clinical data, including primary tumor, radiotherapy and previous treatment options, were collected. Patients received topical 4% formalin irrigation in a clasp-knife position under spinal epidural anesthesia in the operating room. Remission of rectal bleeding and related complications were recorded. Defecation, remission of bleeding, and other symptoms were investigated at follow-up. Endoscopic findings in patients with rectovaginal fistulas were analyzed.

RESULTS: Twenty-four patients (19 female, 5 male) with a mean age of 61.5 ± 9.5 years were enrolled. The mean time from the end of radiotherapy to the onset of bleeding was 11.1 ± 9.0 mo (range: 2-24 mo). Six patients (25.0%) were blood transfusion dependent. The median preoperative Vienna Rectoscopy Score (VRS) was 3 points. Nineteen patients (79.2%) received only one course of topical formalin irrigation, and five (20.8%) required a second course. No side effects were observed. One month after treatment, bleeding cessation was complete in five patients and obvious in

14; the effectiveness rate was 79.1% (19/24). For long-term efficacy, 5/16, 1/9 and 0/6 patients complained of persistent bleeding at 1, 2 and 5 years after treatment, respectively. Three rectovaginal fistulas were found at 1 mo, 3 mo and 2 years after treatment. Univariate analysis showed associations of higher endoscopic VRS and ulceration score with risk of developing rectovaginal fistula.

CONCLUSION: Modified formalin irrigation is an effective and safe method for hemorrhagic CRP, but should be performed cautiously in patients with a high endoscopic VRS.

Key words: Chronic radiation proctitis; Efficacy; Rectal bleeding; Safety; Topical formalin irrigation

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Core tip: The study describes a modified topical formalin irrigation procedure that was well tolerated with long-term effectiveness for refractory hemorrhagic chronic radiation proctitis. The method focused on improving safety and reducing complications. The advantages of the procedure were as follows: protection of internal sphincter (spinal epidural anesthesia and the clasp-knife position provide full anal dilatation instead of dilatation by an anal retractor); protection of proximal normal colonic mucosa (Foley catheter inserted into the proximal sigmoid cavity to prevent damage from formalin backflow); targeting of the lesion area; and well-controlled volume and irrigation time.

Ma TH, Yuan ZX, Zhong QH, Wang HM, Qin QY, Chen XX, Wang JP, Wang L. Formalin irrigation for hemorrhagic chronic radiation proctitis. *World J Gastroenterol* 2015; 21(12): 3593-3598 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3593.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3593>

INTRODUCTION

Radiotherapy is an essential treatment modality for pelvic malignancies such as gynecological, rectal and prostate cancer. However, chronic radiation proctitis (CRP) is a common and severe complication in these patients, with 29%-51% developing rectal hemorrhage following pelvic radiotherapy^[1,2]. The underlying causes for this type of complication include endarteritis obliterans and progressive submucosal fibrosis^[3,4].

Refractory hemorrhagic CRP is difficult to manage^[2,5-9], but previous successful experience in treating cystitis has led to the use of formalin as a treatment option^[10]. Topical formalin application has been extensively studied, and most results show that it is a simple, safe and effective way to treat hemorrhagic CRP. Formalin can be applied by direct instillation or by endoscopy-

guided placement of formalin-soaked gauze^[10-14]. Formalin acts only on the superficial mucosa, which results in rapid deterioration of mucosal blood flow and superficial coagulation necrosis^[3,15,16].

Despite the efficacy, high complication rates after formalin application have been reported, such as anal pain, rectal stricture, and incontinence^[17]. De Parades *et al.*^[18] conducted a prospective study and suggested that formalin should be used carefully in cases of radiation-induced anorectal stricture, previous anal incontinence, and anal cancer. Is it not clear if topical formalin application causes local ischemia of the rectal wall that results in complications such as stricture and fistulas. There are no studies evaluating the safety of application methods, or identifying which patients may not be suitable for this treatment. Therefore, we conducted a retrospective study of patients treated for refractory hemorrhagic CRP, with a focus on improving the safety and reducing the complications of formalin irrigation.

MATERIALS AND METHODS

Patient selection and data collection

Patients receiving a modified method of topical formalin irrigation for refractory hemorrhagic CRP between August 2007 and November 2013 at the Sixth Affiliated Hospital of Sun Yat-Sen University were enrolled. Exclusion criteria were: (1) patients with large ulcers, mucosal necrosis, or stricture [Vienna Rectoscopy Score (VRS): 4-5 points] due to increased risk of perforation; (2) patients with life-threatening or mild bleeding that could be controlled by medical treatment; (3) patients allergic to formalin; and (4) patients with relapse of a primary tumor. All patients enrolled had grade II-III rectal bleeding according to the Common Terminology Criteria for Adverse Events (CTCAE) 4.0^[19], and had not responded to previous medical treatment such as topical corticosteroids, sucralfate, and 5-aminosalicylic acid. Data encompassing general characteristics, treatment details of the primary malignancy, clinical and endoscopic evaluations, details of topical formalin irrigation, change in rectal bleeding, and potential complications were collected. The study was approved by the Ethical Committee of the Sixth Affiliated Hospital of Sun Yat-Sen University and met the guidelines of the local responsible governmental agency. Due to the retrospective nature of the study, informed consent was waived.

Procedures

All patients received flexible endoscopic evaluation before formalin irrigation and were scored according to VRS criteria^[20] (Table 1). A 30-min water enema was performed, and patients then received topical formalin irrigation in a clasp-knife position under spinal epidural anesthesia in the operating room. First, a

Table 1 Vienna rectoscopy score of endoscopic findings for hemorrhagic chronic radiation proctitis

Score	Congested mucosa	Telangiectasia	Ulceration	Stricture	Necrosis
0	Focal reddening	None	None	None	None
1	Diffuse, nonconfluent	Single	None	None	None
2	Diffuse confluent	Multiple, nonconfluent	None	None	None
3	Any	Multiple, confluent	Micro-ulceration, superficial, < 1 cm ²	None	None
4	Any	Any	Superficial, > 1 cm ²	> 2/3 regular diameter	None
5	Any	Any	Deep ulceration, fistula, perforation	≤ 2/3 regular diameter	Any

The highest grade of any one parameter qualifies for the attribution to one of the given score levels regardless of the grade achieved in any other parameter.

Table 2 Patient demographics *n* (%)

Characteristic	Value
Age, yr	61.5 ± 9.5
Sex, female/male	19/5
Primary cancer	
Cervical	15 (62.5)
Endometrium	3 (12.5)
Prostatic	3 (12.5)
Rectal	2 (8.3)
Cervical and ovarian	1 (4.2)
Total irradiation dosage ¹ , Gy	75 (44-97)
Concomitant chemotherapy	13 (54.2)
History of abdominopelvic operation	13 (54.2)
History of acute radiation proctitis	19 (79.2)
Time from the end of radiotherapy to bleeding, mo	11.1 ± 9.0
Duration of bleeding, mo	10.6 ± 8.0
Grade of bleeding, CTCAE v 3.0	
II	20 (83.3)
III	4 (16.7)
Preoperative hemoglobin, g/L	107.6 ± 16.4
Transfusion dependent	6 (25.0)
Preoperative VRS	3 (1-5)

¹Data from 15 patients (9 received radiotherapy in other centers). VRS: Vienna rectoscopy score.

Foley catheter was inserted into the proximal sigmoid cavity to prevent formalin backflow. Then, 10-20 mL 4% formalin was topically irrigated towards the rectal hemorrhagic surface of the mucosa under direct observation for 0.5-3.0 min until bleeding ceased. A semicircular anal speculum was used to protect the normal mucosa, superficial ulceration, and the anal canal. Finally, water was injected to wash out the remaining formalin. This procedure could be repeated after 1 wk in the absence of obvious cessation of bleeding.

Follow-up

Patients were followed-up by telephone after 1, 3 and 6 mo, and then every year for 5 years after treatment. Defecation was evaluated *via* patients' descriptions at follow-up regarding stool frequencies, existence of tenesmus, fecal incontinence (or sanitary pad use), constipation, and anal pain. Other data recorded included: remission of bleeding (defined as complete cessation, partial remission, unchanged, or worsened), other symptomatic complaints, and subsequent treatments after formalin application. The efficacy

of formalin irrigation was determined 1 mo after treatment.

Statistical analysis

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY, United States). The Shapiro-Wilk test was used to evaluate the normality of continuous variables. Student's *t* test was used to assess normally distributed data (presented as mean ± SD), and a Wilcoxon rank-sum test was performed to assess non-normal distributions (data presented as median and range). Pearson's χ^2 test was performed to compare categorical variables. Two-sided *P* < 0.05 was considered as statistically significant.

RESULTS

Demographics

Thirty-one patients were initially enrolled. Twenty-four patients were followed-up for a median 20 mo (Table 2); seven patients did not complete follow-up evaluation (survival status unknown). Primary tumors included cervical, endometrial, prostatic, rectal and ovarian cancer. Patients with gynecological cancer received external radiotherapy, intracavity irradiation, or both. Patients with prostate or rectal cancer received external radiotherapy or intensity-modulated radiotherapy.

Ten patients (41.7%) had other symptoms such as abdominal pain, anal pain, fecal urgency, tenesmus, or diarrhea. The linear extent of proctitis was 3-15 cm from the anal verge. Thirteen patients (54.2%) had proximal proctitis change below 7 cm: 11 patients had distal proctitis and associated sigmoiditis was observed in two patients (20 cm from the anal verge). All patients received medical treatments for bleeding such as topical corticosteroids (*n* = 10), sucralfate (*n* = 15), hemostatics (*n* = 18), and traditional Chinese medicine (*n* = 10). No patients were on anticoagulant treatment. No recurrence or metastasis was found for primary pelvic malignancies during follow-up.

Modified topical formalin irrigation

Topical formalin irrigation was performed on 20 patients in a clasp-knife position under spinal epidural anesthesia, and four patients were treated in the lithotomy position under general or regional anesthesia

Table 3 Demographic and clinical parameters of three patients with rectal fistulas after topical formalin irrigation

Parameter	Case 1	Case 2	Case 3
Preoperative VRS score	5	5	4
Preoperative ulcer score	3	2	2
Formalin concentration	4%	4%	4%
Time of formalin exposure	30 s	1 min	2 min
Courses of formalin irrigation	1	1	1
Postoperative VRS score	Unknown	5	5
Time from the end of radiotherapy to fistula formation, mo	3	20	1
Therapy for rectal fistula	Diversion	Parks operation	Parks operation

VRS: Vienna rectoscopy score.

because they could not tolerate a clasp-knife position due to their age. The duration of irrigation was 2 min for the majority ($n = 19$) of procedures, and ranged from 30 s to 5 min. All but one of the procedures were performed with 4% formalin (2% was used in 1 case). Nineteen patients received only one course of topical formalin irrigation, and five required a second course. No adverse effects were reported after treatment.

Efficacy of formalin irrigation

One month after treatment, five patients showed complete cessation of bleeding, 14 presented only minor bleeding, and five still had bleeding, for a 79.1% (19/24) effectiveness rate. Three months after treatment, 6/22 patients presented with bleeding. One year after treatment, 5/16 patients complained of persistent bleeding, which was reduced to 1/9 patients and 0/6 patients at 2 and 5 years after treatment, respectively.

Rectovaginal fistulas and associated endoscopic findings

A total of three rectovaginal fistulas (RVFs) were reported at 1, 3 and 2 years after treatment (Table 3). Surgical interventions were conducted for these patients, including fecal diversion ($n = 1$) and Parks' operation (a sphincter-saving operation involving resection of the rectum and perianal anastomosis of healthy colon to the anal canal)^[21] ($n = 2$). Univariate analysis of endoscopic findings showed that a higher VRS and ulceration score were significantly related to risk of RVF ($P < 0.05$) (Table 4).

DISCUSSION

The incidence of radiation proctitis after radiotherapy for pelvic malignant tumors ranges from 5% to 20%^[22]. Rectal bleeding is the most common symptom, and refractory bleeding is problematic. To help control rectal bleeding in CRP patients, sucralfate, 5-aminosalicylic acid, metronidazole, steroids and fatty acids have been used, albeit with inconsistent and unsatisfactory

Table 4 Univariate analysis of endoscopic findings after topical formalin irrigation

Variable	With fistula	Without fistula	<i>P</i> value ¹
VRS scores	5 (4-5)	3 (1-5)	0.012
Friable mucosa	1 (0-1)	1 (0-3)	0.374
Telangiectasis	2 (1-2)	2 (1-3)	0.231
Ulcer	0 (0-5)	4 (0-5)	0.022
Necrosis	0 (0-5)	0 (0-5)	0.556

¹Wilcoxon rank sum test; data are presented as median (range).

results^[23]. Endoscopic treatment with argon plasma coagulation (APC) is an effective and popular option for patients with refractory hemorrhagic CRP; however, it can result in rectal ulceration, stricture, bowel perforation, and RVF^[10]. In our clinical center, we used APC for several patients with hemorrhagic CRP. The results were satisfactory for patients with limited lesion surface areas, but for patients with massive areas of telangiectasia, complications such as anal pain, tenesmus and rectal stricture were observed.

Topical application of formalin is considered a safe and effective treatment for hemorrhagic CRP, with comparable efficacy and fewer complications than APC^[24]. In this study, modified formalin irrigation was effective in 79.1% of patients after 1 mo, which is similar to previous studies^[10,12-14,25,26]. In our series, 18 (75.0%) patients reported rapid reduction in rectal bleeding at 2 d after treatment. Endoscopic findings revealed decreased severity of telangiectasia, reflecting the reduction of mucosal blood flow after formalin irrigation. Furthermore, bleeding only persisted in one patient after 2 years. However, resolution of rectal bleeding cannot be entirely attributed to formalin irrigation, because it may reduce spontaneously when the fibrosis of the rectal wall progresses^[27].

With an emphasis on safety, we modified the formalin irrigation procedure, resulting in a low rate of complications compared with previous studies^[17,18,28]. The modified method protects the internal sphincter; spinal epidural anesthesia and the clasp-knife position provided full anal dilatation rather than violent dilatation by an anal retractor. The proximal normal colon mucosa is also protected from formalin backflow by insertion of a Foley catheter, which can reduce risks of colitis and peritonitis. We used a semicircular anal speculum for visual formalin irrigation, therefore, the lesion could be directly targeted, thus preventing damage to the normal rectal mucosa, superficial ulcerations, anal canal, and perianal skin. The volume and time of irrigation are well controlled, thus further reducing the risk of unintended damage. Although three patients developed RVF, these may have been a result of the natural progression of CRP. Our analysis shows that high endoscopic VRS and high ulceration score are associated with risk of RVF. Therefore,

we suggest that formalin irrigation should be more cautiously performed in these patients. Whether formalin damages the deep rectal wall remains an open question^[18].

There were several limitations to this study that may have produced potential bias, including the retrospective nature of the study, small sample size, and empirical therapy. Additional prospective randomized controlled trials are therefore needed to confirm the efficacy and safety of this method.

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COMMENTS

Background

Chronic radiation proctitis (CRP) occurs in 5%-20% of patients receiving radiotherapy for pelvic malignant tumors such as cervical and prostatic cancer. The most common symptom is rectal bleeding, which is difficult to manage. Medical and endoscopic treatments have been tried, with unsatisfactory results. Argon plasma coagulation (APC) is a popular and effective option for CRP, but results in complications. Thus, it is critical to introduce new treatment options to reduce potential complications.

Research frontiers

Recently, different methods utilizing formalin for hemorrhagic CRP have been reported, including direct instillation and endoscopy-guided insertion of formalin-soaked gauze, with efficacy comparable to that of APC. However, these methods still result in complications. In this study, a new method is presented for application of formalin with improved safety and few complications.

Innovations and breakthroughs

In this series, a modified method of topical 4% formalin irrigation was introduced and shown to be effective and well tolerated for refractory hemorrhagic CRP. This procedure offers protection of the internal sphincter and proximal normal colon mucosa, and targets the lesion area with well-controlled irrigation volume and time.

Applications

By improving the safety of topical formalin irrigation and targeting the CRP lesion, complications such as anal pain, rectal stricture, and incontinence can be reduced. Moreover, the efficacy for controlling rectal bleeding can be enhanced, and thus improve quality of life.

Terminology

The underlying causes of CRP are endarteritis obliterans and progressive submucosal fibrosis due to radiotherapy. Formalin acts on the superficial mucosa of the rectum and results in the rapid deterioration of mucosal blood flow, which leads to superficial coagulation necrosis to resolve bleeding.

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This is an important experience for what is sometimes a difficult problem. The method of application is repeated 3 times in the text (abstract, methods and in the discussion). Installation or irrigation may be more appropriate than application for the technique. Other treatment options like endoscopic plasma coagulation should be discussed.

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Retrospective Study

Poor prognosis for hepatocellular carcinoma with transarterial chemoembolization pre-transplantation: Retrospective analysis

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Abstract

AIM: To investigate whether transarterial chemoembolization (TACE) before liver transplantation (LT) improves long-term survival in hepatocellular carcinoma (HCC) patients.

METHODS: A retrospective study was conducted among 204 patients with HCC who received LT from January 2002 to December 2010 in PLA General Hospital. Among them, 88 patients received TACE before LT. Prognostic factors of serum α -fetoprotein (AFP), intraoperative blood loss, intraoperative blood transfusion, disease-free survival time, survival time with tumor, number of tumor nodules, tumor size, tumor number, presence of blood vessels and bile duct invasion, lymph node metastasis, degree of tumor differentiation, and preoperative liver function were determined in accordance with the Child-Turcotte-Pugh (Child) classification and model for end-stage liver disease. We also determined time of TACE before transplant surgery and tumor recurrence and metastasis according to different organs. Cumulative survival rate and disease-free survival rate curves were prepared using the Kaplan-Meier method, and the log-rank and χ^2 tests were used for comparisons.

RESULTS: In patients with and without TACE before LT, the 1, 3 and 5-year cumulative survival rate was $70.5\% \pm 4.9\%$ vs $91.4\% \pm 2.6\%$, $53.3\% \pm 6.0\%$ vs $83.1\% \pm 3.9\%$, and $46.2\% \pm 7.0\%$ vs $80.8\% \pm 4.5\%$, respectively. The median survival time of patients with and without TACE was 51.857 ± 5.042 mo vs 80.930 ± 3.308 mo ($\chi^2 = 22.547$, $P < 0.001$, $P < 0.05$). The 1, 3 and 5-year disease-free survival rates for patients with and without TACE before LT were $62.3\% \pm 5.2\%$ vs

98.9% \pm 3.0%, 48.7% \pm 6.7% *vs* 82.1% \pm 4.1%, and 48.7% \pm 6.7% *vs* 82.1% \pm 4.1%, respectively. The median survival time of patients with and without TACE before LT was 50.386 \pm 4.901 mo *vs* 80.281 \pm 3.216 mo ($\chi^2 = 22.063$, $P < 0.001$, $P < 0.05$). TACE before LT can easily lead to pulmonary or distant metastasis of the primary tumor. Although there was no significant difference between the two groups, the chance of metastasis of the primary tumor in the group with TACE was significantly higher than that of the group without TACE.

CONCLUSION: TACE pre-LT for HCC patients increased the chances of pulmonary or distant metastasis of the primary tumor, thus reducing the long-term survival rate.

Key words: Liver transplantation; Hepatocellular carcinoma; Transarterial chemoembolization; Long-term survival rate; Disease-free survival rate

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Core tip: Hepatocellular carcinoma (HCC) has a high prevalence in China. Patients always have a long-term history of liver cirrhosis, varying degrees of portal hypertension symptoms, and the tumor volume exceeds the Milan criteria when they receive treatment. Whether it is necessary to adopt transarterial chemoembolization (TACE), which is more commonly used in China pre-transplantation, when the patients in waiting state. This study assessed the influence of preoperative TACE on long-term survival in liver transplantation (LT). TACE pre-LT in patients with HCC increased the chances of pulmonary or distant metastasis of the primary tumor, thus reducing long-term survival.

Li HL, Ji WB, Zhao R, Duan WD, Chen YW, Wang XQ, Yu Q, Luo Y, Dong JH. Poor prognosis for hepatocellular carcinoma with transarterial chemoembolization pre-transplantation: Retrospective analysis. *World J Gastroenterol* 2015; 21(12): 3599-3606 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3599.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3599>

INTRODUCTION

About 600000 people die of hepatocellular carcinoma (HCC) annually, making it one of the most common malignancies worldwide^[1]. Only about 10%-30% of patients have the opportunity for surgery^[2], which is mainly liver resection and liver transplantation (LT). There is considerable controversy about whether liver transplant patients in the waiting period should receive other adjuvant therapy, such as transarterial chemoembolization (TACE) and radiofrequency

ablation (RFA)^[3-10]. Some researchers propose that TACE before LT can help patients meet the strict Milan criteria and thus prolong postoperative long-term survival^[11]. However, other researchers propose that it is inappropriate to perform adjuvant treatment before LT for patients with HCC because spread and metastasis may occur at the liver puncture site^[12,13]. It has also been reported that incomplete local treatment may cause sarcomatous changes in patients with HCC^[14]. Kim *et al.*^[15] reported that treatment before LT has a significant impact on the prognosis if the volume ratio of the active tumor is $> 10\%$. Wong *et al.*^[16] reported that the chance of necrosis was $< 75\%$ in five patients, due to local/non-local recurrence, and the use of RFA, TACE, or cisplatin gelatin injection.

In China, the tumor volume in the vast majority of patients with HCC already exceeds the Milan criteria when they receive treatment. Also, these patients always have a long-term history of liver cirrhosis, and varying degrees of portal hypertension symptoms are also seen in some patients. For such patients, there is still no consensus about whether it is necessary to adopt adjuvant therapy, such as TACE, which is more commonly used in China before transplantation. Thus, a retrospective analysis was carried out to assess the influence of preoperative TACE on long-term survival rates in liver transplant patients.

MATERIALS AND METHODS

Clinical data

We analyzed retrospectively 204 patients with HCC and cirrhosis receiving LT from January 2002 to December 2010.

Patients constitution

Two hundred and four patients met the inclusion criterion, 180 male and 24 female, with ages ranging from 31 to 68 years [average: 50 (50.23 \pm 7.88) years]. There were 88 and 116 patients in the groups with and without TACE, respectively. The duration of follow-up was 96 mo with a follow-up rate of 100%. All donors made voluntary donations, including 155 cases of cardiac death and 49 living donors. No donor was a condemned donor. The PLA General Hospital Ethics Committee reviewed and supervised the entire donation process to ensure compliance with these requirements.

Preoperative examination

Preoperative diagnosis of HCC relied on imaging examinations such as ultrasound, computed tomography (CT) and enhanced nuclear magnetic resonance imaging (MRI). All patients received radionuclide bone scans, chest CT, and/or positron emission tomography-CT examinations to exclude extrahepatic metastases.

Inclusion criteria

Patients who had no lung or abdominal metastases or lymph node metastasis, as determined by chest and abdominal CT or MRI examination; patients who had no metastases, as determined by whole-body bone scan; patients whose HCC was confirmed by postoperative pathology; and patients who had long-term follow-up.

Exclusion criteria

Patients who had serious perioperative complications or died, and patients who died of non-liver-related diseases.

Patient information

Clinical data included age (years), serum α -fetoprotein (AFP), blood loss, intraoperative blood transfusion, disease-free survival time, and survival time with tumor. AFP was divided into four groups: normal range ≤ 20 ng/L, 20-400 ng/L, 400-1000 ng/L, and > 1000 ng/L. Tumor size was divided into three groups: meeting the Milan criteria (≤ 5 cm), complying with the UCSF criteria (5-8 cm), and beyond the UCSF criteria (≥ 8 cm). The degree of tumor differentiation was in accordance with the Edmondson grade: level I was highly differentiated (G1); II and III were moderately differentiated (G2); and IV was low differentiation (G3). Grades I and II were classified as well-differentiated, and III and IV as poorly differentiated. Numbers of lesions, determined on the basis of preoperative CT, MRI, and pathological examinations, were divided into single and multiple groups. Preoperative liver function was determined in accordance with the Child classification and the model for end stage liver disease (MELD). Based on time of TACE from transplant surgery, the patients were divided into three groups: ≤ 1 mo, 1-3 mo, and ≥ 3 mo. Survival state was divided into live and dead groups. Liver surgery was divided into whole liver transplantation and living donor transplantation groups. The patients were divided into tumor recurrence and no tumor recurrence groups. According to postoperative complications, patients were divided into four groups: no complications, biliary complications, vascular complications, and infection. Tumor recurrence and metastasis were divided according to different organs: liver, lung and bone.

Liver resection and tumor histopathological evaluation

Numbers of tumor nodules, tumor size, tumor number, presence of blood vessels and bile duct invasion, and lymph node metastasis were determined. Tumor grade was determined according to the standard Edmondson classification. For multiple tumors, total tumor diameter was the maximum diameter of each tumor.

Postoperative immunosuppressive and other therapies

Cyclosporine A + mycophenolate mofetil + methylprednisolone (CsA + MMF + Pred) and tacrolimus (FK506

+ MMF + Pred) were used as the main postoperative regimens. Intravenous infusion of hepatitis B immunoglobulin, combined with oral administration of lamivudine and other antiviral drugs, was adopted after surgery to prevent any recurrence of hepatitis B.

TACE before LT

Femoral artery puncture was carried out according to the Seldinger method. A catheter was inserted into the target vessel branches (usually the hepatic or superior mesenteric artery and other arteries) for imaging to determine the location, size, number, and artery of the tumor. After catheter infusion chemotherapy, a vessel was inserted to the supplying arteries (proper hepatic artery, left and right hepatic artery, or branch artery) of the tumor, and iodized oil, chemotherapy drug suspensions, and gelatin sponge were injected for thrombosis.

Follow-up

All patients were followed up after surgery. Methods of follow-up included inpatient and outpatient follow-up and telephone calls. Liver transplant patients were checked monthly in the first 6 mo, and then every 2 mo in the second 6 mo. After that, patients were examined every 3-6 mo. The patient's condition changes were recorded, and routine examinations of blood, liver and kidney function, blood drug concentrations, and qualitative and quantitative examination of hepatitis B virus were performed. Tumor recurrence and metastasis were monitored by AFP, CT, color Doppler ultrasound, chest radiography, whole-body bone ECT, and other tests. The time and location of tumor recurrence and the time and cause of death were recorded.

Statistical methods

Measurement data are presented as mean \pm SD or median, and the *t* test or χ^2 test was used for comparisons between the groups. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare the survival probability. The cumulative survival rate was expressed as rate \pm SE, and $P < 0.05$ was considered to indicate a significant difference. SPSS version 16.0 (SPSS, Chicago, IL, United States) was used.

RESULTS**Patient data**

Basic data for the 204 cases are shown in Table 1. There were 88 cases with preoperative TACE and 116 without preoperative TACE. The basic data of the two groups were similar. There was no statistically significant difference between age, Child classification or MELD score, AFP values, intraoperative blood loss, or intraoperative autotransfusion between the groups. However, disease-free survival time and survival time with tumor between the two groups did show

Table 1 Statistical data of the groups with and without preoperative transarterial chemoembolization

Demographics	TACE (<i>n</i> = 88)	No TACE (<i>n</i> = 116)	<i>t</i>	<i>P</i> value
Age (yr)	49.34 ± 8.06	50.90 ± 7.70	-1.401	0.163
Child	7.33 ± 2.69	7.23 ± 2.46	0.267	0.789
MELD	8.36 ± 7.16	8.07 ± 5.74	0.324	0.747
IBL (mL)	2944.32 ± 4008.34	2528.79 ± 2681.56	0.886	0.377
IOA (mL)	1910.34 ± 2902.87	1539.53 ± 2532.91	0.972	0.332
AFP (ng/L)	3439.08 ± 6623.56	1922.59 ± 5180.01	1.835	0.068
Disease-free survival time (mo)	20.77 ± 17.52	32.79 ± 22.63	-4.131	< 0.001 ^a
Survival time with tumor (mo)	1.88 ± 3.10	0.68 ± 2.10	3.278	0.001 ^a

^a*P* < 0.05, TACE *vs* no TACE. TACE: Transarterial chemoembolization; Child: Child-Turcotte-Pugh classification; MELD: Model for end-stage liver disease; AFP: α-fetoprotein; IBL: Intraoperative blood loss; IOA: Intraoperative autotransfusion.

Table 2 Categorical data of the groups with and without preoperative transarterial chemoembolization treatment

Variables and stratification	TACE (<i>n</i> = 88)	No TACE (<i>n</i> = 116)	χ^2	<i>P</i> value
Sex			0.352	0.553
Male	79	101		
Female	9	15		
MELD			5.269	0.072
< 15	80	104		
15-25	5	12		
> 25	3	0		
Child			0.207	0.902
A	56	77		
B	14	18		
C	18	21		
Operation method			1.874	0.171
CDLT	71	84		
LDLT	17	32		
AFP			14.141	0.003 ^a
< 20	21	47		
≤ 400	22	38		
400-1000	18	9		
> 1000	27	22		
Tumor recurrence			21.983	< 0.001 ^a
No	46	96		
Yes	42	20		
Complications			3.064	0.382
No	66	97		
Complication of bile duct	8	9		
Complication of vessel	7	6		
Infection	7	4		

^a*P* < 0.05, TACE *vs* no TACE. TACE: Transarterial chemoembolization; Child: Child-Turcotte-Pugh classification; MELD: Model for end-stage liver disease; AFP: α-fetoprotein; CDLT: Cadaveric donor liver transplantation; LDLT: Living donor liver transplantation.

significant differences (*P* < 0.05).

Categorical data between the groups with and without preoperative TACE

Tumor recurrence and AFP in the two groups showed

Table 3 Postoperative pathological data for the two groups of patients

Variables and stratification	TACE (<i>n</i> = 88)	No TACE (<i>n</i> = 116)	χ^2	<i>P</i> value
Tumor size			6.569	0.037 ^a
Within Milan criteria	38	70		
Within UCSF criteria	22	24		
Beyond UCSF criteria	28	22		
Vascular invasion			12.138	< 0.001 ^a
No	48	90		
Yes	40	26		
Tumor Edmondson grade			2.521	0.112
Well-moderate	57	87		
Moderate-poor	31	29		
Number of tumors			15.632	< 0.001 ^a
Solitary	38	82		
Multiple	50	34		

^a*P* < 0.05, TACE *vs* no TACE. TACE: Transarterial chemoembolization.

significant differences (*P* < 0.05). The differences in sex, MELD score, Child classification, surgical approach, and postoperative complications between the two groups were not significant (*P* > 0.05). The results are shown in Table 2.

Postoperative pathological data

Postoperative pathological data for the two groups of patients are shown in Table 3. Tumor size was divided into three groups: meeting the Milan criteria (≤ 5 cm), complying with the UCSF criteria (5-8 cm), and beyond the UCSF criteria (≥ 8 cm). The degree of tumor differentiation was in accordance with the Edmondson grade: a moderate-high differentiation group and a moderate-low differentiation group; the two groups showed no significant difference (*P* > 0.05). The size, number, and vascular invasion in the two groups showed significant differences (*P* < 0.05).

Influence of TACE before LT on long-term and disease-free survival

The 1, 3 and 5-year cumulative survival rates of the patients with TACE before LT were 70.5% ± 4.9%, 53.3% ± 6.0%, and 46.2% ± 7.0%, respectively, while for patients without TACE treatment before LT, they were 91.4% ± 2.6%, 83.1% ± 3.9%, and 80.8% ± 4.5%, respectively. The median survival times of the patients with and without TACE before LT were 51.857 ± 5.042 and 80.930 ± 3.308 mo, respectively ($\chi^2 = 22.547$, *P* < 0.001, *P* < 0.05), and the cumulative survival curves are shown in Figure 1. The 1, 3 and 5-year disease-free survival rates of the patients with TACE before LT were 62.3% ± 5.2%, 48.7% ± 6.7%, and 48.7% ± 6.7%, respectively, while for patients without TACE treatment before LT, they were 98.9% ± 3.0%, 82.1% ± 4.1%, and 82.1% ± 4.1%, respectively, and cumulative disease-free survival curves are shown in Figure 2. The median survival times of patients with and without TACE treatment

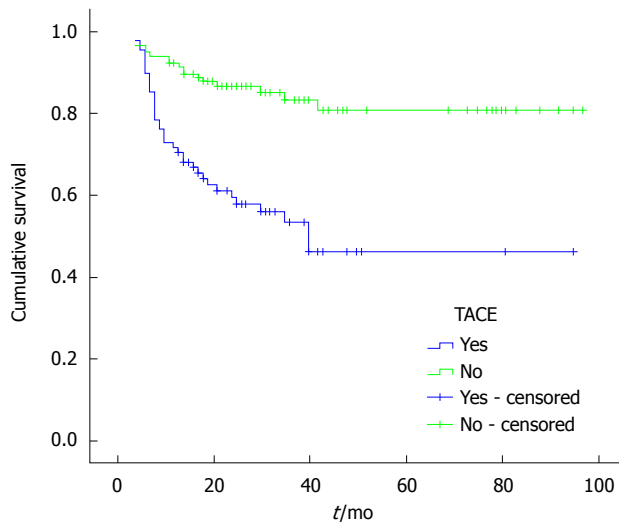


Figure 1 Cumulative survival curves of patients plotted using the Kaplan-Meier method. Green line: Cumulative survival curve of the 116 patients with TACE before LT; Blue line: Cumulative survival curve of the 88 patients without TACE before LT. The cumulative survival rates of the two groups showed a significant difference ($P < 0.05$, log-rank test). TACE: Transarterial chemoembolization; LT: Liver transplantation.

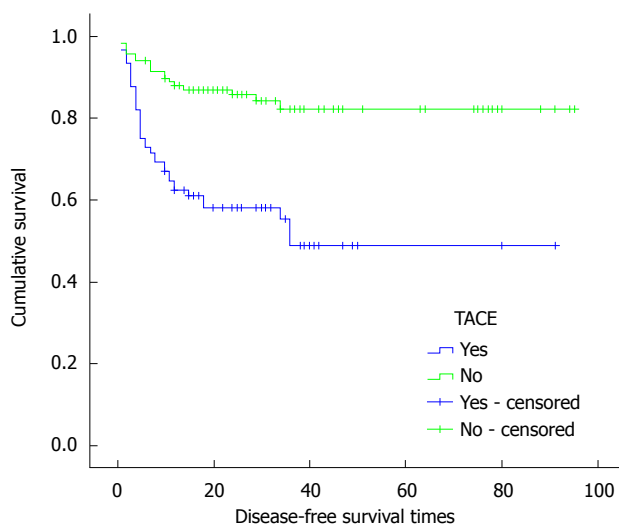


Figure 2 Cumulative disease-free survival curves of patients plotted using the Kaplan-Meier method. Green line: Cumulative disease-free survival curve of the 116 patients with TACE before LT; Blue line: Cumulative disease-free survival curve of the 88 patients without TACE before LT. The cumulative disease-free survival rates of the two groups showed a significant difference ($P < 0.05$, log-rank test). TACE: Transarterial chemoembolization; LT: Liver transplantation.

before LT were 50.386 ± 4.901 and 80.281 ± 3.216 mo, respectively ($\chi^2 = 22.063$, $P < 0.001$, $P < 0.05$).

Influence of time of TACE from LT on location and recurrence of the tumor

The 62 patients with tumor recurrence were divided into three groups according to tumor size: those meeting the Milan criteria, complying with UCSF criteria, and beyond the UCSF criteria. Based on the time of TACE treatment from transplant surgery, the

Table 4 Comparison of the groups with and without transarterial chemoembolization treatment at different times from the liver transplantation

	TACE			No TACE	χ^2	P value
	≤ 1 mo	1-3 mo	≥ 3 mo			
Milan criteria	2	1	8	5	16	0.001 ^a
UCSF criteria	1	1	7	6	15	0.002 ^a
BUCSF criteria	8	9	5	9	31	< 0.001 ^a

^a $P < 0.05$, TACE *vs* no TACE. TACE: Transarterial chemoembolization.

patients were divided into three groups: ≤ 1 , 1-3 and ≥ 3 mo. As seen in Table 4, the results showed that the groups with and without TACE treatment showed significant differences ($P < 0.05$).

Tumor recurrence and metastases were divided according to different organs: liver, lung and bone, and multiple metastases. The results showed that for the groups beyond UCSF with and without TACE, $\chi^2 = 10.459$, $P = 0.015$ and $P < 0.05$, respectively, while for the groups meeting the Milan criterion and UCSF with and without TACE treatment, there was no significant difference ($P > 0.05$). As seen in Table 5, the number of cases with pulmonary and distant metastasis in the TACE groups was higher than that of the groups without TACE.

DISCUSSION

LT as a curative and effective therapy for HCC can remove the tumor and cirrhosis of the liver tissue and avoid malignant changes in residual disease in liver tissue^[17,18]. However, whether TACE before LT is suitable for patients with HCC is still controversial. Grasso and others have suggested that preoperative adjuvant therapy has no effect on the recurrence rate when the Milan criteria are not followed^[19]. Yao *et al*^[20] have proposed that proper preoperative treatment is necessary for patients exceeding the Milan criteria. Roayaie and others have suggested that preoperative TACE combined with postoperative doxorubicin chemotherapy has satisfactory therapeutic effects for large liver cancers and LT^[21]. Aggressive preoperative treatment may have a positive role in reducing neoplasm stage, and thus reduce the rate of tumor recurrence.

Our results suggest that sex, MELD score, Child classification, surgical approach, postoperative complications, and other general clinical data in the groups with and without TACE made no significant difference. The 1-, 3- and 5-year cumulative survival rates of the patients with and without TACE were significantly different ($\chi^2 = 22.547$, $P < 0.001$, $P < 0.05$). The cumulative disease-free survival rates of the two groups were also significantly different ($\chi^2 = 22.063$, $P < 0.001$, $P < 0.05$). These results indicate that preoperative TACE had no positive role on the

Table 5 Tumor recurrence and metastasis in the groups with and without transarterial chemoembolization

	TACE				No TACE				χ^2	P value
	Liver	Lung	Bone	M	Liver	Lung	Bone	M		
Milan criteria	2	7	0	2	1	3	0	1	0.019	0.990
UCSF criteria	0	7	0	2	1	3	0	2	2.083	0.353
BUCSF criteria	3	11	0	8	5	3	1	0	10.459	0.015 ^a

^aP < 0.05, TACE *vs* no TACE. TACE: Transarterial chemoembolization.

long-term or disease-free survival rates for HCC. Indeed, on the contrary, they may reduce the lifetime of the patients. Docaens and others have proposed that preoperative TACE does not prolong long-term survival^[3]. Sarasin and others noted that only when liver cancer patients waited > 8 mo for LT did the tumor show unfavorable prognostic factors for LT^[22]. If the waiting time for LT is 1-2 mo, TACE may have no effect. If the waiting time for LT is a few months, TACE may make the tumor remain at the earliest state, and thus may be beneficial in controlling tumor growth during the period of waiting for a donor.

In the 62 cases of patients with tumor recurrence, tumor size (meeting the Milan criteria, complying with UCSF criteria, and beyond the UCSF criteria) and the time of TACE from transplant surgery (≤ 1 , 1-3 and ≥ 3 mo) in the groups with and without TACE did show significant differences ($\chi^2 = 16.0$, $P = 0.001$; $\chi^2 = 15.0$, $P = 0.002$; $\chi^2 = 31.0$, $P < 0.001$, $P < 0.05$, respectively). Tumor recurrence and metastasis were divided according to different organs: liver, lung and bone, and multiple metastases. Comparisons between the TACE and non-TACE groups showed that TACE before LT may cause pulmonary and distant metastases. The number of cases meeting the USCF criteria in the TACE group was higher than in the non-TACE group, although there was no significant difference between them. For patients beyond the USCF criteria, larger tumors were more common in the TACE group, and comparison of the two groups showed a significant difference ($P < 0.05$). The mechanism remains unclear.

The current study shows that residual liver cancer cells and normal liver tissue undergo gene expression changes after TACE; that is, TACE can promote angiogenesis factor expression in residual tumor cells. Animal experiments have demonstrated that after TACE, in tumor remnants, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, microvascular density, and proliferative activity of the non-embolized tumor cells increased^[23]. An *et al*^[24] found that VEGF levels in the peripheral blood of patients with HCC after TACE increased, and expression of VEGF by cancer cells and noncancerous liver cells in surgical specimens from a two-stage operation after TACE also increased. Li *et al*^[25] found that part of the non-embolized liver appears to undergo compensatory hyperplasia and increased proliferative activity after

TACE. The possibility for recurrence and metastasis of residual HCC cells increases after TACE. The remnants of normal liver tissue may lead to recurrence of HCC, due to cirrhosis and an increase in compensatory hyperplasia and proliferative activity after TACE. Furthermore, recent studies have shown that the establishment of collateral circulation after TACE is a major factor for HCC recurrence and metastasis, which may lead to more vulnerable growth in other parts of the tumor cells with high metastatic potential. The specific mechanisms remain to be studied further. However, at least based on current findings, there is no conclusive evidence that preoperative TACE before LT can prolong long-term survival after LT in patients with HCC^[21,26-33].

In conclusion, for the patients with HCC before LT, especially for patients who can undergo LT in < 3 mo, preoperative TACE is not appropriate and may result in lung and/or distant metastases. However, whether this conclusion applies to other transplant centers needs further study.

COMMENTS

Background

Hepatocellular carcinoma (HCC) has a high prevalence in Asian countries, particularly China. These patients always have a long-term history of liver cirrhosis, varying degrees of portal hypertension symptoms, and the tumor volume exceeds the Milan criteria when they receive treatment. So, there is still no consensus about whether it is necessary to adopt transarterial chemoembolization (TACE), which is more commonly used in China before liver transplantation (LT). This study assessed the influence of preoperative TACE on long-term survival rates in liver transplant patients.

Research frontiers

A retrospective study included 204 patients with HCC and cirrhosis who received LT from January 2002 to December 2010. The numbers of patients in the groups with and without TACE before LT were 88 and 116, respectively. All patients were in the PLA General Hospital.

Innovations and breakthroughs

The results demonstrated TACE before LT for patients with HCC had no positive influence on long-term survival or disease-free survival rates, but increased the chances of pulmonary or distant metastasis of the primary tumor, thus reducing the long-term survival rate.

Applications

Preoperative TACE before LT for patients with HCC had no positive influence on long-term or disease-free survival rates, however, it increased the chances of pulmonary or distant metastasis of the primary tumor.

Peer-review

This study aimed to evaluate outcomes whether TACE treatment before LT can improve long-term survival rates in HCC patients. The results are interesting and suggest that preoperative TACE treatment before LT for the patients with HCC had no positive influence on long-term survival or disease-free survival

rates, however increased the chances of pulmonary metastasis or distant metastasis of the primary tumor. It can be reduced the long-term survival rate.

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Retrospective Study

Endoscopic ultrasound-guided deep and large biopsy for diagnosis of gastric infiltrating tumors with negative malignant endoscopy biopsies

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deep and large biopsy technique under the guidance of endoscopic ultrasound (EUS) for diagnosis of gastric infiltrating tumors with negative malignant endoscopy biopsies.

METHODS: From January 2009 to March 2014, 36 patients in whom gastric infiltrating tumors had been diagnosed by EUS received negative results for malignancy after endoscopic biopsies. The deep and large biopsy technique combined bite-on-bite technique with or without endoscopic mucosal resection (EMR) to obtain submucosal tissue from lesions. EUS was used to select the appropriate biopsy sites. If the lesion protruded into the cavity, EMR was performed for removal of the overlying mucosa and then bite-on-bite technique was conducted in the resected area to obtain submucosal tissue. If the lesion appeared to be flat or was difficult to lift by injection, the bite-on-bite technique was directly used.

RESULTS: Twenty-eight of the 36 patients were treated by EMR followed by bite-on-bite technique, while 8 patients only underwent bite-on-bite technique. Histological results showed 23 of the 36 lesions were poorly differentiated adenocarcinomas, 2 diffuse large B cell lymphomas, 4 mucosa-associated lymphoid tissue-type lymphomas, and 7 undiagnosed. The deep and large biopsy technique provided a definitive and conclusive diagnosis in 29 (80.6%) of the 36 patients. The 12 gastric linitis plastica and 6 lymphoma patients received chemotherapy and avoided surgery. Minor oozing of blood in 2 mucosal resection wounds was managed by argon plasma coagulation and in 5 cases after deep biopsies by epinephrine (0.001%). Neither severe hemorrhage nor perforation occurred in any patient.

CONCLUSION: The deep and large biopsy technique is superior to ordinary endoscopic biopsy for achieving an accurate diagnosis of gastric infiltrating tumors.

Abstract

AIM: To assess the diagnostic yield and safety of a

This procedure guided by EUS is an effective and safe diagnostic method for gastric infiltrating tumors in which endoscopic biopsy results were negative for malignancy.

Key words: Endoscopic ultrasonography; Endoscopic biopsy; Diagnosis; Gastric linitis plastica; Gastric lymphoma

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Core tip: The diagnosis of gastric infiltrating tumors is challenging, which is often delayed due to negative endoscopic and histological tests. We for the first time investigated the deep and large biopsy technique for diagnosis of gastric infiltrating tumors with negative malignant endoscopy biopsies. This biopsy technique combined bite-on-bite technique with or without endoscopic mucosal resection. Endoscopic ultrasound was used to select the thickest site for biopsy. The biopsy provided a definitive and conclusive diagnosis in 29 (80.6%) of the 36 patients. Neither severe hemorrhage nor perforation occurred. It is an effective and safe diagnostic method for gastric infiltrating tumors with negative endoscopy biopsies.

Zhou XX, Pan HH, Usman A, Ji F, Jin X, Zhong WX, Chen HT. Endoscopic ultrasound-guided deep and large biopsy for diagnosis of gastric infiltrating tumors with negative malignant endoscopy biopsies. *World J Gastroenterol* 2015; 21(12): 3607-3613 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3607.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3607>

INTRODUCTION

The most common gastric infiltrating tumors are gastric linitis plastica (GLP) and gastric lymphoma. GLP is a diffuse, infiltrating carcinoma characterized by thickening and rigidity of the stomach wall. Generally, GLP infiltrates the submucosal layer without destroying the structure of the stomach wall, and thus specific findings in the mucosal layer are insufficient for making a diagnosis^[1,2]. Few patients are curable because at diagnosis the tumor is frequently advanced, with invasion of neighboring organs or distant metastasis^[2]. On the other hand, primary gastric lymphoma comprises only 5% of gastric malignant tumors^[3]. Most gastric lymphomas originate in the submucosa, and diagnosis *via* gastroscopy and forceps biopsy is often difficult^[4]. The distinction between gastric lymphoma and GLP is also important for the treatment.

Endoscopic ultrasound (EUS) is a reliable nonsurgical technique for diagnosis and staging of gastrointestinal malignancies. The EUS examination has become an integral part of the pre-therapeutic evaluation in patients suspected of submucosal tumors of the upper gastrointestinal tract^[5,6]. EUS can be used to ascertain

the echogenicity, location, size, and depth of lesions and perigastric lymph nodes that are the diagnostic criteria for GLP or gastric lymphoma^[4,7]. On EUS images, GLP is more likely to feature a pattern of vertical spread, while horizontal spread is more typical of gastric lymphoma^[4,8]. Although some lesions have distinctive EUS characteristics, using these diagnostic criteria alone to distinguish lymphoma from GLP is inadequate. Consequently, tissue sampling is necessary to establish a conclusive diagnosis.

Specimens obtained from a standard endoscopic biopsy rarely provide a confirmative diagnosis because lesions in the submucosa are difficult to reach with forceps. To clarify the diagnosis, repeated biopsies or deep biopsy is required. It has been reported that the bite-on-bite technique is effective and safe for subepithelial lesions^[9], but the number of cases was limited and lesions did not appear to be hypervascular or under a thick overlying epithelium.

Endoscopic mucosal resection (EMR), which recently has been widely applied for the treatment of early stomach cancer, may be useful in the diagnosis of GLP and gastric lymphoma^[10]. EMR can obtain a larger tissue specimen and therefore may increase the rate of positive diagnostic findings compared with conventional biopsy. However, the procedure is associated with an increased risk of complications, including perforation and bleeding^[11]. Performing EMR under the guidance of EUS may reduce operational risk and complications. However, no systematic study of EMR combined with bite-on-bite technique for diagnosis of gastric infiltrating tumors has been reported.

In the present study, we retrospectively investigated the safety and efficacy of EMR and bite-on-bite technique under the guidance of EUS for diagnosis of gastric infiltrating tumors that had been determined nonmalignant through endoscopic biopsy.

MATERIALS AND METHODS

From January 2009 to March 2014 in our department, 36 patients (19 men, average age 53.5 years, age range: 31-77 years) suspected of gastric infiltrating tumors underwent deep and large biopsies guided by EUS. All patients had undergone ordinary biopsies 2 to 4 times and pathology showed negative results. During routine endoscopic examinations, among the 36 patients, 6 were asymptomatic, while 11, 6, 6, 4 and 3 patients presented with abdominal pain, gastrointestinal tract hemorrhage, abdominal circumference, obstruction, and mass, respectively. They provided informed consent for deep and large biopsies. Therapy, pathology, and image data were extracted. The institutional review board at Zhejiang University approved this study.

All patients received deep and large biopsies under the guidance of EUS. A 12-MHz probe (GF-UM 2R, Olympus, Tokyo, Japan) and two-channel endoscope

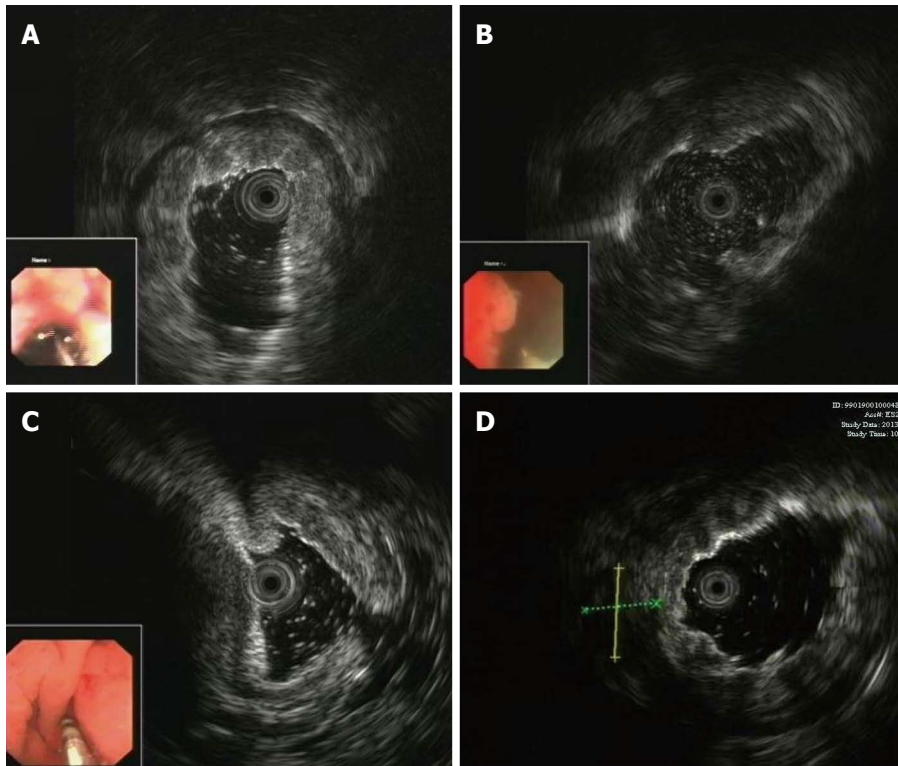


Figure 1 Endoscopic ultrasound characteristics of gastric infiltrating tumors. A: Invaded muscularis propria and the first three blurred sonographic layers; B: Invaded serosal layer; C: Ascites around gastric wall; D: Perigastric lymph node.

(GIF-2T240, Olympus, Tokyo, Japan) were used for ultrasonographic study. The lesion was scanned after filling the stomach with water. By EUS, the location, echogenicity, and infiltrated depth of tumors were characterized, and the maximum thickness of the gastric wall, perigastric lymph nodes, and ascites were noted.

EUS was used to select the thickest site for biopsy. If the lesion protruded into the cavity, EMR was performed for removal of the overlying mucosa and then bite-on-bite technique was conducted in the resected area to obtain submucosal tissue. If the lesion appeared to be flat or was difficult to lift by injection, the bite-on-bite technique was directly used. EMR was performed with a conventional electrosurgical snare (FD-IU, Olympus, Tokyo, Japan) and an electrosurgical unit (VIO 200D, ERBE, Tübingen, Germany). The lesion was lifted by submucosal injection of indigo carmine (0.002%) and epinephrine (0.001%), and the mucosa was then resected. The bite-on-bite technique was performed as previously reported^[11] using a biopsy forceps with needle (Radial jaw 3 standard capacity, Boston Scientific). Each bite was directly taken from top of the previous bite in an attempt to burrow into the lesion. Two to eight bites per lesion were performed for every patient. All specimens were sent for pathologic study, some of which were assayed by immunohistochemistry. Procedural risks and complications such as perforation and hemorrhage were recorded.

RESULTS

Thirty-six patients were examined using EUS, and gastric infiltrating tumors were diagnosed. The lesions were diffusely located in 13 cases, and in 10, 5, 5, and 3 cases located in the body and antrum, fundus and cardia, body only, and antrum only, respectively. EUS showed that the lesion site had been replaced by a hypoechoic or medium-echoic thickened gastric wall. In 24 of the 36 lesions, the muscularis propria was invaded and the first three sonographic layers were blurred or even indistinguishable and absent (Figure 1A), while in the remaining 12 lesions the five sonographic layers were invaded (Figure 1B). The maximum full thickness of the stomach wall ranged from 10 mm to 29 mm, with an average of 16.3 mm. Perigastric lymph nodes were seen in 3 patients and perigastric ascites in 6 patients (Figure 1C and 1D). In 2 patients, both perigastric lymph nodes and perigastric ascites were found.

The deep and large biopsy procedure was performed under the guidance of EUS to determine appropriate biopsy sites (Figures 2 and 3). The choice of EMR or bite-on-bite technique was based on the endoscopic results. Twenty-eight of the 36 patients underwent combined EMR and bite-on-bite technique (Figure 3), while the remaining 8 patients were given bite-on-bite technique alone (Figure 2). Minor oozing of blood in 2 mucosal resection wounds was managed by argon plasma coagulation (APC) and in 5 cases

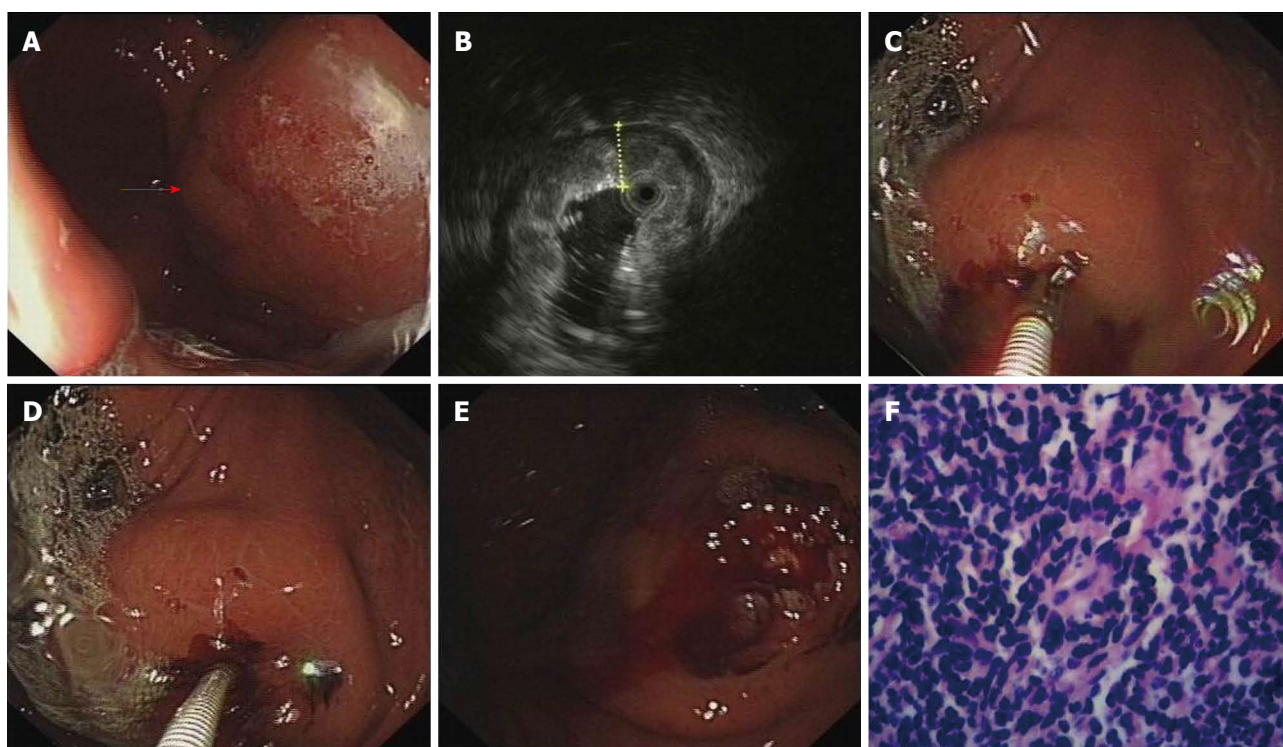


Figure 2 Endoscopic ultrasound-guided bite-on-bite technique. A and B: A gastric infiltrating tumor diagnosed by endoscopic ultrasound (EUS) (red arrow); C: Bite-on-bite technique performed after EUS localization; D: The bite is taken on top of the previous one to obtain valid tissue; E: Postoperative wound; F: Histology confirmed lymphoma (HE staining, $\times 400$).

after deep biopsies by epinephrine (0.001%). None of the patients suffered from severe hemorrhage or perforation.

Postoperative histological results showed that 23 of the 36 lesions were poorly differentiated adenocarcinomas (including 9 signet-ring cell carcinomas), 2 diffuse large B cell lymphomas, 4 mucosa-associated lymphoid tissue-type lymphomas, and 7 of unknown type. The diagnostic yield of the bite-on-bite technique was 6 (75%) of 8, whereas that of EMR with bite-on-bite technique was 23 (82.2%) of 28. The deep and large biopsy technique provided a definitive diagnosis in 29 (80.6%) of the 36 patients (Table 1). Based on the systemic assessment, 3 GLP patients underwent surgery. Twelve unresected GLP and 6 lymphoma patients received chemotherapy, and 5 GLP patients received both surgery and chemotherapy. Three GLP patients refused treatment. Five patients with negative results underwent surgery and the pathologic results showed poorly differentiated adenocarcinomas. Two patients without a definitive diagnosis were confirmed as adenocarcinomas by endoscopy biopsies at the 3- and 6-mo follow-up, respectively.

DISCUSSION

Making a diagnosis of gastric infiltrating tumors is challenging, and is often delayed due to false negative endoscopic and histological tests^[1,2,12]. These tests can be false negative because lesions in the submucosa

are beyond the reach of conventional-sized forceps. In cases of GLP, the false-negative rate with endoscopic mucosal forceps biopsy can be as high as 55.9%^[13]. In our department, from January 2009 to March 2014 there were at least 36 patients with gastric infiltrating tumors determined by EUS, with negative malignant endoscopy biopsies. It has been shown that, for diagnosis of GLP, the accuracy of computed tomography (74.6%, 44/59) was significantly higher than that of gastroscopy (44.1%, 26/59; $P < 0.001$), yet this is still not effective enough for making a clear diagnosis^[13].

EUS may be a viable pre-surgical diagnostic method, increasing diagnostic accuracy and safely predicting gastric infiltrating tumors on the basis of endosonographic characteristics^[4,14]. According to a prospective multicenter study by Rösch *et al.*^[15], EUS had a sensitivity of 64% and a specificity of 80% in differentiating between malignant and benign submucosal tumors. However, the differential diagnosis between GLP and gastric lymphoma is not an easy task. In the present study, EUS showed that in all patients the lesion site had been replaced by a hypoechoic or medium-echoic thickened gastric wall. The invaded sonographic layered structures were blurred or even indistinguishable. Although some of these lesions had distinctive classifiable EUS features, endosonographic criteria alone were inadequate and could not confirm a clear diagnosis.

For tissue acquisition of gastric submucosal lesions,

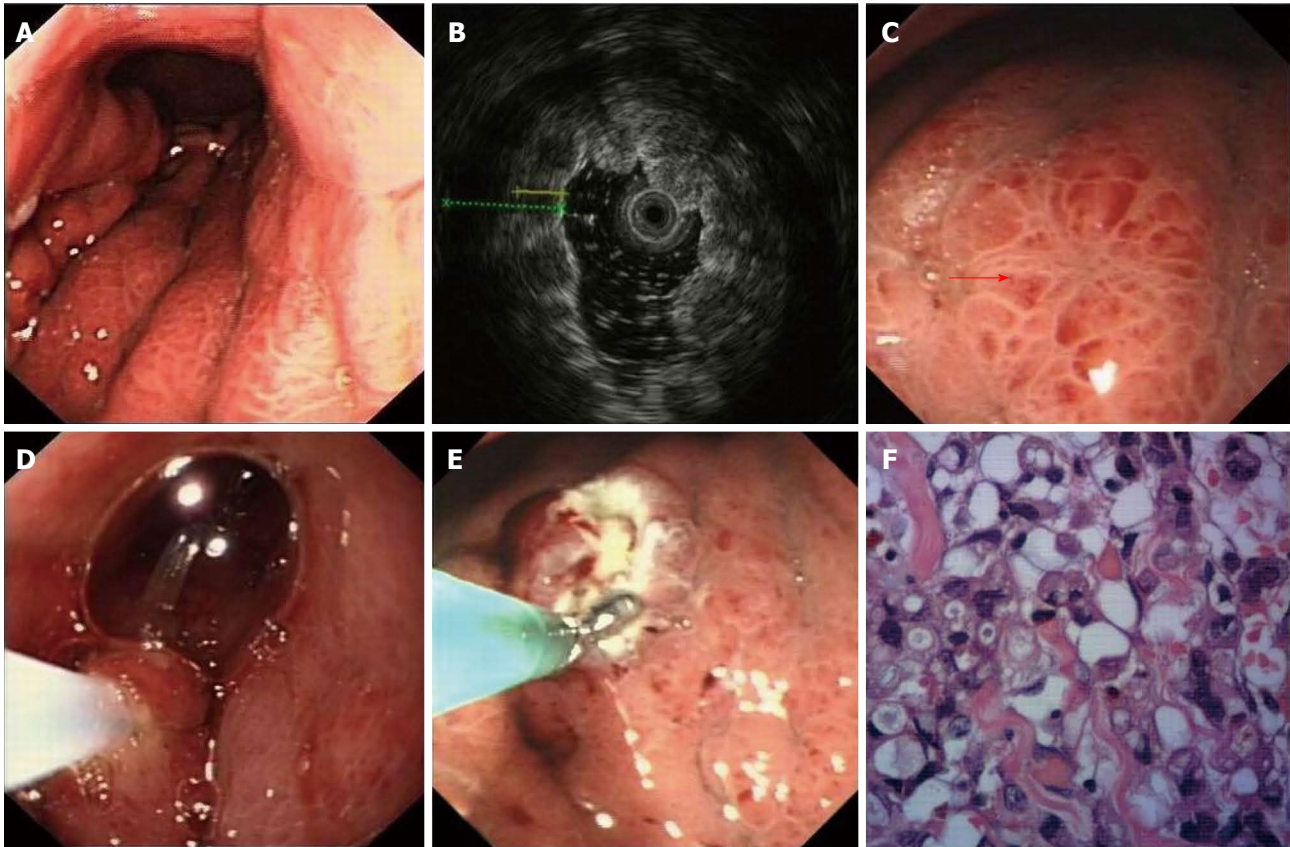


Figure 3 Endoscopic mucosal resection combined with bite-on-bite technique under the guidance of endoscopic ultrasound. A and B: A gastric infiltrating tumor diagnosed by endoscopic ultrasound (EUS); C: The thickest site was selected for biopsy after EUS localization (red arrow); D: The mucosa was then resected; E: Bite-on-bite technique was performed in the resection area; F: Histology confirmed adenocarcinoma with poor differentiation (HE staining $\times 400$).

a variety of deep and large techniques have been developed, such as jumbo biopsy, EUS-guided fine needle aspiration (EUS-FNA), endoscopic submucosal resection, endoscopic submucosal dissection and the bite-on-bite technique^[11,16-20]. It was reported that EUS-FNA provided a definitive diagnosis for sub-epithelial lesions in 14 (45.1%) of 31 patients, while the rate of a clearly definitive diagnosis using the jumbo biopsy forceps was 76 (58.9%) of 129 patients^[17]. According to a retrospective study by Cantor *et al.*^[11], for the evaluation of sub-epithelial tumors the diagnostic yield was 17% (4/23) using the jumbo forceps and 87% (20/23) for endoscopic resection. However, these studies were performed with many limitations. EUS-FNA is not reliable for obtaining valid tissue and may be inadequate or inaccurate for diagnosis^[16,18,20]. The use of jumbo forceps or EMR may increase the surface area of the tissue sample, but does not significantly increase its depth^[11], and there are procedural risks and complications such as perforation and hemorrhage^[17]. The bite-on-bite technique for deep biopsy of the stomach wall yields valid submucosal tissues, which may increase the accuracy rate for clear and positive diagnoses. Nevertheless, gastric infiltrating tumors usually have a thickened epithelium which may limit the use of bite-on-bite technique. In this study, we assessed the diagnostic yield of combined EMR and

bite-on-bite technique for gastric infiltrating tumors that had received negative results for malignancy *via* endoscopy biopsies. Based on the endoscopic results, 28 of 36 patients were treated by combined EMR and bite-on-bite technique, and the other 8 patients only underwent bite-on-bite technique. The deep and large biopsy technique provided a definitive and confirmative diagnosis in 29 (80.6%) of the 36 patients.

Before planning an appropriate therapy, definitive pathology tests and results are essential for diagnosis of gastric infiltrating tumors. In the present study, based on the systemic assessment patients given a definite diagnosis underwent individualized treatment. The 12 unresected GLP and 6 lymphoma patients received chemotherapy and avoided surgery. Thus, deep and large biopsy technique helps to improve decision making in the management of gastric infiltrating tumors.

Previous studies showed that deep and large biopsy techniques for submucosal lesions have been associated with a relative risk of complications, mainly hemorrhage and perforation^[11,19-22]. To reduce the complication rate, Cantor *et al.*^[11] proposed that endoscopic resection should be performed in obviously symptomatic patients (*e.g.*, with gastrointestinal bleeding or abdominal pain or obstruction). In asymptomatic patients, it should be limited to lesions

Table 1 Diagnosis by deep and large biopsy techniques in the 36 gastric infiltrating tumors with negative malignant endoscopy biopsies *n* (%)

	<i>n</i>	GLP	Gastric lymphoma	No diagnosis
Bite-on-bite technique	8	5 (62.5)	1 (12.5)	2 (25.0)
EMR combined with bite-on-bite technique	28	18 (64.3)	5 (17.9)	5 (17.9)
Total	36	23 (63.9)	6 (16.7)	7 (19.4)

EMR: Endoscopic mucosal resection; GLP: Gastric linitis plastica.

that are either malignant or suspected malignant. EUS-guided biopsy has the potential to reduce the complication rate. In our study, EUS was used to select the correct excision site. Deep and large biopsies were performed successfully in all the 36 patients. Minor oozing of blood occurred in 7 patients, which was easily managed with APC or epinephrine (0.001%) during the procedure. Neither severe hemorrhage nor perforation occurred in any patient.

In conclusion, the deep and large biopsy is superior to ordinary biopsy in its ability to achieve an accurate and positive diagnosis of gastric infiltrating tumors. The procedure guided by EUS is an effective and safe diagnostic method for gastric infiltrating tumors with negative endoscopy biopsies, and is also suitable for the diagnosis of other sub-epithelial lesions of the gastrointestinal tract. In addition, diagnostic results can provide key information for decision making in the management of gastric infiltrating tumors.

COMMENTS

Background

The diagnosis of gastric infiltrating tumors is challenging, which is often delayed due to false negative endoscopic and histological tests. These tests can be false negative because lesions in the submucosa are beyond the reach of conventional-sized forceps.

Research frontiers

For tissue acquisition of gastric submucosal lesions, a variety of deep and large techniques have been developed. However, the procedures are associated with an increased risk of complications, including perforation and bleeding. In this study, we for the first time investigated the diagnostic yield and safety of a deep and large biopsy technique under the guidance of endoscopic ultrasound (EUS) for diagnosis of gastric infiltrating tumors with negative malignant endoscopy biopsies.

Innovations and breakthroughs

The deep and large biopsy was superior to ordinary biopsy in its ability to achieve an accurate and positive diagnosis of gastric infiltrating tumors. Patients received deep and large biopsies under the guidance of EUS without severe complications. The diagnostic results help patients to make decisions in their next therapies.

Applications

EUS-guided deep and large biopsy technique is an effective and safe diagnostic method for gastric infiltrating tumors with negative endoscopy biopsies, and is also suitable for the diagnosis of other sub-epithelial lesions of the gastrointestinal tract. Diagnostic results can provide key information for decision making in the management of gastric infiltrating tumors.

Terminology

The deep and large biopsy technique combined bite-on-bite technique with or without endoscopic mucosal resection (EMR) technique to obtain submucosal

tissue from lesions. The bite-on-bite technique was used with each bite taken from the top of the previous bite in an attempt to burrow into the lesion. Two to eight bites per lesion were performed for every patient.

Peer-review

The authors present important research findings and the paper is timely as findings demonstrate improvement in diagnostic approach. The article describes an elegant solution by the combination of EUS and EMR as well as "inkwell" biopsy.

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Retrospective Study

Cholecystectomy does not significantly increase the risk of fatty liver disease

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Ethics approval: This study was reviewed by Nanjing Medical University Institutional Review Board. Ethical approval for this investigation was obtained from the Ethics Committee of Nanjing Medical University.

Informed consent: All participants were informed about the purpose and general procedures of this study and informed consents were signed prior to study enrollment.

Conflict-of-interest: The authors declare that there is no conflict of interest.

Data sharing: No additional unpublished data are available.

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Abstract

AIM: To investigate the relationship between cholecystectomy and fatty liver disease (FLD) in a Chinese population.

METHODS: A total of 32428 subjects who had voluntarily undergone annual health checkups in the Second Affiliated Hospital of Nanjing Medical University from January 2011 to May 2013 were included in this study. Basic data collection, physical examination, laboratory examination, and abdominal ultrasound examination were performed.

RESULTS: Subjects undergoing cholecystectomy were associated with greater age, female sex, higher body mass index, and higher levels of systolic blood pressure, diastolic blood pressure, fasting plasma glucose, total cholesterol, and triglycerides. However, no significant differences were found in high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, albumin, and serum uric acid. The overall prevalence of FLD diagnosed by ultrasonography was high at 38.4%. The prevalence of FLD was significantly higher for subjects who had undergone cholecystectomy (46.9%) than those who had not undergone cholecystectomy (38.1%; χ^2 test, $P < 0.001$). Cholecystectomy was positively associated with FLD (OR = 1.433, 95%CI: 1.259-1.631). However, after adjusting for possible factors associated with

FLD, multivariate regression analysis showed that the association between cholecystectomy and FLD was not statistically significant (OR = 1.096; 95%CI: 0.939-1.279).

CONCLUSION: According to our study results, cholecystectomy may not be a significant risk factor for FLD.

Key words: Cholecystectomy; Fatty liver disease; Relationship; Cross-sectional study

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Core tip: The prevalence of fatty liver disease (FLD) without cholecystectomy was 38.1%, and was up to 46.9% with cholecystectomy, showing an increase in the prevalence of FLD after cholecystectomy. However, no significant association was found between cholecystectomy and FLD after adjusting for multiple related factors. To the best of our knowledge, this is the first study on the correlation between cholecystectomy and FLD in a large Chinese population.

Wang HG, Wang LZ, Fu HJ, Shen P, Huang XD, Zhang FM, Xie R, Yang XZ, Ji GZ. Cholecystectomy does not significantly increase the risk of fatty liver disease. *World J Gastroenterol* 2015; 21(12): 3614-3618 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3614.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3614>

INTRODUCTION

Fatty liver disease (FLD) is a leading cause of chronic liver disease in China^[1], and is classified as nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease according to etiology^[2,3]. Recent population-based epidemiological studies indicate that the median prevalence of FLD in China is 17% (12.5%-27.3%), and approximately 90% of cases appear to be nonalcoholic^[4-8]. FLD may progress to end-stage liver disease and then to steatohepatitis, advanced fibrosis, cirrhosis, and hepatocellular carcinoma^[1,2,8-10]. Although it is not a direct cause of death, FLD is considered a preclinical condition related to cardiovascular disease and other metabolic diseases^[2,9,11,12].

Cholecystectomy is one of the most common surgical procedures due to its curative effect. However, there are many long-term complications associated with this procedure. Whether cholecystectomy is a risk factor for FLD requires further study. According to a recent report^[13], the prevalence of NAFLD has increased possibly due to the metabolic effects of the absence of the gallbladder after cholecystectomy. Cholecystectomy in mice also led to FLD by changing triglyceride metabolism^[14]. This may be interpreted as an alteration in the enterohepatic circulation of

bile acids^[15] and the loss of metabolic activity of the gallbladder mucosa^[16]. However, these reports are not enough to confirm this conclusion. Whether cholecystectomy increases the prevalence of FLD requires more research. In this study, we attempted to determine the association between cholecystectomy and FLD in a large population-based study.

MATERIALS AND METHODS

All participants were informed about the purpose and general procedures of the examination. The Ethics Committee of Nanjing Medical University approved the study protocol and manner of consent.

Study subjects were recruited from participants who had voluntarily undergone annual health checkups at the Second Affiliated Hospital of Nanjing Medical University between January 2011 and May 2013. The analyses were limited to the subjects who underwent abdominal ultrasonography, and those who had full records of anthropometric and biochemical data. The subjects were included if they complied with the following criteria: (1) absence of markers of hepatitis B virus infection (hepatitis B surface antigen) and hepatitis C virus (HCV) infection (anti-HCV antibody); (2) no previous history of liver disease, including fatty liver; and (3) the absence of other factors inducing fatty changes in the liver, including the use of liver damaging drugs, and autoimmune diseases. Finally, a total of 32428 subjects (22463 men and 9965 women) were enrolled.

Clinical examinations were performed by trained staff using standardized procedures. Height and weight were measured using an automatic digital stadiometer. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. After resting for at least ten minutes, systolic blood pressure (SBP), and diastolic blood pressure (DBP) were measured. Venous blood samples were also obtained from subjects following an overnight fast of more than eight hours and alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG), fasting plasma glucose (FPG), albumin, and serum uric acid (SUA) levels were measured using standard techniques. All subjects received abdominal ultrasonography to determine FLD and cholecystectomy combined with a surgical history.

Statistical analyses were performed using SPSS 18.0 software for Windows (SPSS Inc., Chicago, IL, United States). Due to non-normal distribution, continuous variables were expressed as median and interquartile range (25%-75%) due to abnormal distribution of the data. Comparisons between the independent groups were conducted using the Mann-Whitney *U* test. Categorical variables were compared

Table 1 Demographics and clinical characteristics of the subjects according to cholecystectomy

	With cholecystectomy (<i>n</i> = 949)	Without cholecystectomy (<i>n</i> = 31479)	<i>P</i> value
Age (yr)	63 (54-73)	49 (36-59)	< 0.001
Sex (male/female)	447/502	22016/9463	< 0.001
BMI (kg/m ²)	24.26 (22.55-26.65)	23.92 (21.71-26.09)	< 0.001
SBP (mmHg)	131 (121-141)	125 (116-135)	< 0.001
DBP (mmHg)	80 (72-88)	77 (69-86)	< 0.001
FPG (mmol/L)	5.41 (4.97-5.99)	5.04 (4.68-5.47)	< 0.001
TC (mmol/L)	5.07 (4.43-5.74)	4.93 (4.34-5.57)	< 0.001
TG (mmol/L)	1.28 (0.92-1.74)	1.14 (0.82-1.67)	< 0.001
HDL cholesterol (mmol/L)	1.28 (1.10-1.51)	1.28 (1.09-1.50)	0.240
LDL cholesterol (mmol/L)	2.88 (2.37-3.42)	2.88 (2.39-3.40)	0.987
ALT (U/L)	19 (14-26)	19 (14-27)	0.132
AST (U/L)	20 (17-24)	20 (17-24)	0.237
GGT (U/L)	22 (16-33)	22 (15-36)	0.243
Albumin (g/L)	47.7 (45.8-49.3)	47.7 (45.6-49.6)	0.835
SUA (mmol/L)	327 (265-379)	329 (275-386)	0.070
FLD (yes/no)	445/504	12003/19476	< 0.001

The data are expressed as the median (IQR) due to abnormal distribution. Mann-Whitney *U* and χ^2 test were performed. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; TC: Total cholesterol; TG: Triglycerides; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase; SUA: Serum uric acid; FLD: Fatty liver disease.

Table 2 Logistic regression analysis of odds ratios for fatty liver disease relative to cholecystectomy

	Multivariate regression analysis		
	OR	95%CI	<i>P</i> value
Cholecystectomy (not adjusted)	1.433	1.259-1.631	< 0.001
Cholecystectomy (adjusted ¹)	1.096	0.939-1.279	0.245

¹Data were adjusted for age, gender, BMI, SBP, DBP, FPG, TC, TG, HDL cholesterol, LDL cholesterol, ALT, AST, GGT, albumin, and SUA. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; TC: Total cholesterol; TG: Triglycerides; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase; SUA: Serum uric acid.

using the χ^2 test. Multivariate logistic regression analyses were conducted to assess the odds ratio (OR) for FLD, comparing subjects with cholecystectomy to those without cholecystectomy. *P* < 0.05 (2-tailed) was considered statistically significant.

RESULTS

Of 32428 subjects enrolled in this study, 949 subjects had a history of cholecystectomy. Compared with subjects without cholecystectomy, cholecystectomy was associated with higher age, female sex, higher BMI, and higher levels of SBP, DBP, FPG, TC, and TG. However, between the two groups, no significant differences were found in HDL cholesterol, LDL cholesterol, ALT, AST, GGT, albumin, and SUA (Table 1). These results indicate that cholecystectomized subjects may not have more metabolic abnormalities than those without cholecystectomy, particularly serum lipids and liver function abnormalities.

In this study, the overall prevalence of FLD diagnosed by ultrasonography was high at 38.4%. Using the χ^2 test, the prevalence of FLD was significantly higher for cholecystectomized subjects (46.9%) than those without cholecystectomy (38.1%; χ^2 test, *P* < 0.001). In addition, logistic regression analysis, not considering other risk factors, showed that cholecystectomy was positively associated with FLD (OR = 1.433, 95%CI: 1.259-1.631) (Table 2). In order to avoid the influence of other relevant factors, multiple logistic regression analysis was further performed to investigate the ORs for FLD with cholecystectomy. After adjusting for possible factors associated with FLD, multivariate regression analysis showed that the association between cholecystectomy and FLD was not statistically significant (OR = 1.096; 95%CI: 0.939-1.279). These results may indicate that there is no significant association between cholecystectomy and FLD.

DISCUSSION

Several studies have investigated the positive association between cholecystectomy and FLD^[13,14]. However, in our study, the results indicated that cholecystectomy may not be associated with FLD. Although the prevalence of FLD in the cholecystectomized subjects was higher than those without cholecystectomy, no significant association was found between cholecystectomy and FLD after adjusting for multiple related factors.

In a population from the United States, the prevalence of NAFLD with cholecystectomy was 48.4%. In addition, the prevalence was higher than that in subjects with (34.4%) or without gallstone disease (17.9%). Controlling for numerous factors associated with both NAFLD and gallstone disease, multivariate-adjusted analysis confirmed the association between

NAFLD and cholecystectomy (OR = 2.4; 95%CI: 1.8-3.3), indicating that cholecystectomy may be a risk factor for NAFLD^[13]. In our study, the prevalence of FLD without cholecystectomy was 38.1%, and was up to 46.9% with cholecystectomy, showing an increase in the prevalence of FLD after cholecystectomy. However, no significant association was found when adjusted by age, gender, BMI, SBP, DBP, FPG, TC, TG, HDL cholesterol, LDL cholesterol, ALT, AST, GGT, albumin, and SUA. These metabolic factors are closely related to FLD. Therefore, to study the relationship between cholecystectomy and FLD, these metabolic factors should be considered.

The previous study from the United States^[17] found that subjects with cholecystectomy were more likely to have elevated serum ALT and GGT, and was associated with the development of cirrhosis. However, the levels of serum liver enzymes, including ALT, AST and GGT, were not changed after cholecystectomy in our study. There is no reasonable explanation for this, although different eating habits and different BMI between subjects in the United States and China may play a part. In addition, several possible biological mechanisms were analyzed. Following cholecystectomy, bile is continuously secreted into the duodenum, and the bile acid pool circulates more quickly, exposing the liver to a greater flux of bile acids^[18-20]. The gallbladder mucosa is metabolically active, secreting and absorbing compounds to and from the bile and loss of the gallbladder provides another possible mechanism for the increased risk of NAFLD following cholecystectomy^[13].

However, our results are not entirely consistent with other studies, which might be due to the following shortcomings. First, information regarding smoking and drinking status, physical activity, and the surgical approach for cholecystectomy (open or laparoscopic) were not available, and these factors may act as confounding variables in the association between cholecystectomy and FLD. Further longitudinal studies should consider these confounding factors. Second, the causal relationship between cholecystectomy and FLD might not be reliable using a cross-sectional analysis, although we ensured all cholecystectomy subjects had no FLD before cholecystectomy. Thus, a prospective cohort study may be more convincing. Finally, the study subjects were recruited at one provincial hospital and therefore may not represent the entire community. Fortunately, these limitations may be partly balanced by the benefits of using a large population-based sample, particularly the avoidance of ascertainment bias which occurs in studies of selected patients.

In conclusion, in 32428 Chinese subjects, cholecystectomy might not be a significant risk factor for FLD according to our findings. Further studies should be performed to verify these results due to the limitations of our study.

COMMENTS

Background

Fatty liver disease (FLD) is considered a preclinical condition related to cardiovascular disease and other metabolic diseases. It has been reported that the prevalence of nonalcoholic fatty liver disease (NAFLD) has increased possibly due to the metabolic effects of the absence of the gallbladder after cholecystectomy. However, whether cholecystectomy is a risk factor for FLD remains to be further studied.

Research frontiers

In a population from the United States, the prevalence of NAFLD with cholecystectomy was 48.4%. The prevalence was higher than that in subjects with (34.4%) or without gallstone disease (17.9%). Controlling for numerous factors associated with both NAFLD and gallstone disease, multivariate-adjusted analysis confirmed the association between NAFLD and cholecystectomy (OR = 2.4; 95%CI: 1.8-3.3), indicating that cholecystectomy may be a risk factor for NAFLD.

Innovations and breakthroughs

In this study, the prevalence of FLD without cholecystectomy was 38.1%, and was up to 46.9% with cholecystectomy, showing an increase in the prevalence of FLD after cholecystectomy. However, no significant association was found between cholecystectomy and FLD after adjusting for multiple related factors.

Applications

In this study, cholecystectomy may not be a significant risk factor for FLD in our 32428 Chinese subjects. Further studies should be performed to verify the results due to the limitations of our study.

Peer-review

As the manuscript point out, FLD was classified as NAFLD and alcoholic liver disease. The author analysis the data by combining the two types, and concluded that cholecystectomy does significantly increase the risk of FLD.

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Clinical Trials Study

Scintigraphy in laryngopharyngeal and gastroesophageal reflux disease: A definitive diagnostic test?

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Author contributions: Falk GL originated the study, conducted the pH and manometry studies and contributed to the manuscript; Beattie J and Ing A contributed patients to the study, formulated the hypothesis and were involved in manuscript preparation; Falk SE collated the data and entered it into the spreadsheet and interviewed patients; Magee M, Burton L and Van der Wall H designed the scintigraphic study, interpreted the data and helped with statistical analysis and manuscript preparation.

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Abstract

AIM: To investigate the utility of scintigraphic studies in predicting response to laparoscopic fundoplication (LF) for chronic laryngopharyngeal reflux symptoms.

METHODS: Patients with upper aero-digestive symptoms that remained undiagnosed after a period of 2 mo were studied with conventional pH and manometric studies. Patients mainly complained of cough, sore throat, dysphonia and globus. These patients were imaged after ingestion of 99m-technetium diethylene triamine pentaacetic acid. Studies were quantified with time activity curves over the pharynx, upper and lower oesophagus and background. Late studies of the lungs were obtained for aspiration. Patients underwent LF with post-operative review at 3 mo after surgery.

RESULTS: Thirty four patients (20 F, 14 M) with an average age of 57 years and average duration of symptoms of 4.8 years were studied. Twenty four hour pH and manometry studies were abnormal in all patients. On scintigraphy, 27/34 patients demonstrated pharyngeal contamination and a rising or flat pharyngeal curve. Lung aspiration was evident in 50% of patients. There was evidence of pulmonary aspiration in 17 of 34 patients in the delayed study (50%). Pharyngeal contamination was found in 27 patients. All patients with aspiration showed pharyngeal contamination. In the 17 patients with aspiration, graphical time activity curve showed rising activity in the pharynx in 9 patients and a flat curve in 8 patients. In those 17 patients without pulmonary aspiration, 29% (5 patients) had either a rising or flat pharyngeal graph. A rising or flat curve predicted aspiration with a positive predictive value of 77% and a negative predictive value

of 100%. Over 90% of patients reported a satisfactory symptomatic response to LF with an acceptable side-effect profile.

CONCLUSION: Scintigraphic reflux studies offer a good screening tool for pharyngeal contamination and aspiration in patients with gastroesophageal reflux disease.

Key words: Laryngopharyngeal reflux; pH studies; Oesophageal manometry; Gastroesophageal reflux disease; Lung aspiration; Scintigraphy; Cough

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Core tip: Scintigraphic studies offer a good screening tool for patients with gastroesophageal reflux disease (GERD) who are suspected of laryngopharyngeal reflux (LPR) and lung aspiration. Such studies can predict the response to fundoplication. Although the application for this study was in a highly selected group who underwent fundoplication for LPR, the results have been equally valid in over 700 unselected patients with suspected GERD. The technique however requires careful attention to detail for acquisition parameters, particularly with the volume of liquid into which the tracer is introduced.

Falk GL, Beattie J, Ing A, Falk SE, Magee M, Burton L, Van der Wall H. Scintigraphy in laryngopharyngeal and gastroesophageal reflux disease: A definitive diagnostic test? *World J Gastroenterol* 2015; 21(12): 3619-3627 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3619.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3619>

INTRODUCTION

Gastro-oesophageal reflux disease (GERD) has a number of protean manifestations that make it difficult to diagnose and treat. In 2006, the Montreal Consensus Group defined GERD as "a condition which develops when the reflux of stomach contents causes troublesome symptoms or complications". The range of symptoms includes cough, sore throat and atypical chest pain and other apparent non-oesophageal symptoms. A proportion of patients may be asymptomatic even with significant acid reflux^[1,2]. Furthermore, asymptomatic physiological episodes of GERD are a daily manifestation, further complicating the diagnosis^[3].

While traditionally understood GERD (heartburn and regurgitation) is common, it has a different symptom profile to laryngopharyngeal reflux (LPR). The connection between GERD and LPR is however a contentious issue which has been canvassed in both the editorial format^[4] and refereed publication^[5]. These publications, especially the editorial by Spechler^[4]

rightly point out that not all chronic cough is due to GERD, as approximately half the patients treated for LPR do not have evidence of acid-reflux on pH monitoring^[5]. We examined the connection between GERD and LPR in a small, selected population of patients referred for laparoscopic fundoplication as treatment for chronic cough and suspected pulmonary aspiration of refluxate. These patients had been carefully investigated and had established GERD by the standard criteria of pH and manometric studies. Detecting LPR and pulmonary aspiration are the two major blind spots of the accepted approach using pH and manometry, with some evidence that impedance studies may help with detecting LPR^[6,7]. We examined the utility of scintigraphic reflux studies in the diagnosis of standard GERD, LPR and in the direct visualization of pulmonary aspiration of tracer during these studies.

MATERIALS AND METHODS

Population and clinical data

Patients were extracted from a research database for either proven or suspected GERD which had been approved by the Institutional Ethics Committee (LNR/12 CRGH/248). Consecutive patients undergoing laparoscopic fundoplication for suspected LPR disease on the basis of abnormal pH/manometry studies was extracted from this database. All patients were considered if they had predominantly upper respiratory tract symptoms that remained undiagnosed after 8 wk of investigation by appropriate specialists. Major upper respiratory tract symptoms documented were cough, sore throat, recurrent throat clearing, voice change, laryngospasm, aspiration, globus and regurgitation. Any history of heartburn regurgitation and dysphagia was also elicited. Because of the severity of continuing extra-oesophageal symptoms despite full medical management and results of standard investigations for GERD as the likely cause of LPR, patients were surgically treated by fundoplication. This is therefore a highly selected group of patients with a high pre-test probability of GERD causing LPR and with a long history of undiagnosed upper respiratory tract symptoms. Patients were reassessed clinically at three months following surgery for the severity of symptoms and/ or the degree of improvement in symptoms. Clinical data was prospectively collected using a standardized proforma before and after surgery and entered into a database.

Scintigraphy, pH studies and manometry were repeated in 5 patients with recurrent symptoms post-operatively.

pH monitoring (2 channel)

Ambulatory 24 h pH monitoring was performed using antimony crystal dual channel catheters (Medtronic, Synectics Medical, Minneapolis, Minnesota, United States) as described elsewhere. Data was recorded

Table 1 Patient symptom profile

Symptoms	Total (<i>n</i> = 34)
GERD	33
Heartburn	24
Regurgitation	23
LPR	33
Chronic cough	25
Voice change	15
Throat clearing/aspiration	11
Sore throat	10
Globus	5
Laryngospasm	2

GERD: Gastroesophageal reflux disease; LPR: Laryngopharyngeal reflux.

with a Digi trapper Mark III recorder (Medtronic, Synectics Medical) and analysed with the Synectics PW esophagram reflux analysis module (Medtronic, Synectics Medical). Abnormal proximal reflux was based on results of previous studies^[8].

Manometry

Stationary manometry was performed using a water perfused dent sleeve 8 channel catheter (Dent Sleeve International, Mississauga, Ontario, Canada) using standard techniques as described elsewhere. Data was recorded using a multichannel recording system (PC polygraph HR Medtronic, Synectics Medical, Minneapolis, Minnesota, United States) and analysed using PolyGram software program (Medtronic, Synectics Medical, Minneapolis, Minnesota, United States). Oesophageal motility was graded as normal, mildly, moderately or severely ineffective oesophageal motility modified from Kahrilas *et al.*^[9,10].

Scintigraphy

Patients were fasted overnight and medications were ceased for the 24 h prior to the test. While upright, patients were positioned in front of a Hawkeye 4 gamma camera (General Electric, Milwaukee, United States) with markers placed on the mandible and over the stomach to ensure the regions of interest were within the field of view of the camera. Patients swallowed 100-150 mL of water with 40-60 MBq of ^{99m}Tc DTPA followed by another 50 mL of water to clear the mouth and oesophagus of radioactivity. Dynamic images of the pharynx, oesophagus and stomach were obtained for 5 min at 15 s per frame into a 64 × 64 matrix. A second 30 min dynamic image was obtained in the supine position immediately following the upright study utilising 30 s frames. Following acquisition of the supine study, the patients were given a further 50 mL of water with 60 MBq of ^{99m}Tc phytate(colloid) followed by 50 mL of water as a flush. Delayed images were obtained at 2 h to assess the presence of aspiration of tracer activity into the lungs. Images were analysed by time activity curves over the

pharynx, upper and lower half of the oesophagus and a background region over the right side of the chest, away from the stomach and oesophagus. Delayed images were analysed by a line profile over the lungs. Time activity curves were graded as showing no GERD, a falling curve, flat or rising curves. We defined the area under the curves (AUC) for the upper oesophagus and oropharynx after subtraction of the background level of activity as the Falk index.

Statistical analysis

Data was analysed by nonparametric statistical methods as much of the analysis was of ordinal data with multiple studies for each patient. Standard ANOVA statistics, Wilcoxon matched pairs test and Pearson correlation coefficient (2 tails) with significance levels of 0.05 were utilised. Cluster analysis of the principal variables was also undertaken to evaluate linkages between 11 key variables. The Statistica V8 software (Statsoft, Oklahoma, United States) package was used for data analysis.

RESULTS

Population and pre-operative clinical data

There were 34 patients (20 F, 14 M) with an average age of 57 years (range: 38-72 years). Proximal LPR symptoms were reported in 33 of 34 patients with one patient having no proximal symptoms, but severe heartburn and sinusitis. GERD was reported in 22 patients. Details of symptoms are provided in Table 1. Average duration of symptoms was 4.8 years (range: 0.5-22 years). All patients underwent laparoscopic fundoplication on the basis of symptoms, supporting tests and failure of best medical management (including double-dose proton pump inhibitor therapy).

Post-operative clinical data

Patients remained on anti-reflux medical therapy for six weeks post-operatively with cessation prior to the 3 mo review. At three months, total control of symptoms was reported in 27 (79%), partial in 4, giving overall improvement in 31 (91%). The rate of dysphagia was unchanged in 12 (44%). Occasional chest pain and bloating was present in 4, reduction in cough frequency in 1 and continued heartburn but eliminated cough in 1. Scintigraphy demonstrated low-grade reflux to the mid-oesophagus but not the pharynx in this patient. Reappearance of cough on stopping PPI occurred in 2 and no symptom resolution in 1 despite normalisation of scintigraphy and 24-h pH monitoring. Two patients were lost to review. Scintigraphy showed recurrent reflux to the pharynx in the 2 former patients and no evidence of reflux in the latter. The pH study was also normal in the latter patient.

Statistical analysis

Cough was the only significant symptom predicting

Table 2 pH studies

pH studies	mean \pm SD	Range	
		Minimum	Maximum
pH distal episodes	128.06 \pm 91.41	8	356
pH distal %total time	16.04 \pm 19.22	5.2	105
pH distal erect exposure	15.68 \pm 18.72	4.2	102
pH distal sup exposure	20.10 \pm 29.35	13	102
pH proximal episodes	31.30 \pm 38.48	11	145
pH proximal %total time	1.59 \pm 2.11	1	9.7
pH prox erect exposure	4.68 \pm 17.65	0	102
pH prox sup exposure	10.52 \pm 29.49	2	102

pharyngeal contamination by scintigraphy ($P = 0.047$) and voice change predicted aspiration by scintigraphy ($P = 0.038$). All symptoms were strongly correlated with pharyngeal contamination by scintigraphy. Cough, laryngospasm and globus were strongly correlated with aspiration (scintigraphy). Change in voice was present in 15 patients, 11 demonstrated aspiration and 13 pharyngeal contamination by scintigraphy.

24 h two channel pH studies

The 24 h pH studies were abnormal in all patients (Table 2). Episodes of reflux/24 h in the distal oesophagus were a mean of 128 and in the upper oesophagus, a mean of 31. The acid reflux exposure time distal was a mean of 16%, and 20% when supine. Reflux exposure time proximal was a mean 1.6%/24 h and 10.5% when supine. There were strong correlations between all symptoms and each of the proximal 24-h pH parameters. Strong correlations were found for heartburn, regurgitation and distal pH results. Strong correlations were found between severity of pH results and pulmonary aspiration by scintigraphy.

Manometry

Lower oesophageal sphincter pressure had a mean \pm SD of 2.94 \pm 5.03 (normal > 18, range: 0-18 mmHg). Oesophageal motility was frequently abnormal and graded as severe IEM in 16, moderate IEM in 4, mild IEM in 5 and normal in 9. Highly significant correlations were found between impaired motility and pulmonary aspiration ($P = 0.000063$), episodes of reflux by scintigraphy ($P = 0.000011$), amplitude of reflux by scintigraphy ($P = 0.0026$) and the Falk index (AUC) ($P = 0.000001$).

Scintigraphic studies

There was evidence of pulmonary aspiration in 17 of 34 patients in the delayed study (50%). Pharyngeal contamination was found in 27 patients. All patients with aspiration showed pharyngeal contamination. In the 17 patients with aspiration, graphical time activity curve showed rising activity in the pharynx in 9 patients and a flat curve in 8 patients. In those 17 patients without pulmonary aspiration, 29% (5 patients) had either a rising or flat pharyngeal graph. A

rising or flat curve predicted aspiration with a positive predictive value of 77% and a negative predictive value of 100%. There was a significant correlation between pharyngeal contamination and aspiration ($P = 0.000$). All patients in the cohort had evidence of gastro-oesophageal reflux by scintigraphy. Significant correlations were found for almost all pH studies and the isotope episodes, and amplitude of reflux in the oesophagus by scintigraphy ($P < 0.01$) but not for proximal supine acid exposure.

Cluster analysis

The hierarchical tree relationship between variables was expressed as a vertical icicle plot of the 11 variables (Figure 1) with single linkages using non-standardised Euclidean distances^[11]. Tight linkages were shown between positive pharyngeal scintigraphy, aspiration by scintigraphy, manometric motility studies and impaired oesophageal motility, proximal total and exposure time.

DISCUSSION

When assessing patients for persistent upper respiratory tract symptoms (predominantly cough) and no cause is immediately apparent, there is accumulated clinical evidence to consider the possibility of LPR. However, approximately 30% of the population in one series of 2000 random cases had a score of over 10 in the reflux symptom index^[12] with 75% also complaining of symptomatic GERD^[13] (Figure 2). The task of assessing GERD in such a vast number of patients is problematic as there would be the need for a relatively invasive algorithm of endoscopy, pH/manometry/ impedance monitoring, ENT examination, respiratory function studies, amongst others. This would incur substantial costs and patient inconvenience. Endoscopy shows abnormalities in less than 50% of patients with reflux disease^[14]. The presence of pepsin in saliva has shown promise as a surrogate marker of GERD that reaches the pharynx^[15,16] but does not indicate lung aspiration of refluxate. A simple non-invasive test is required to assess the presence of significant full-column or proximal oesophageal reflux with potential for pharyngeal contamination and pulmonary aspiration of gastric contents. This particular standardised protocol for scintigraphy has the potential to be such an option.

Two channel 24 h pH monitoring provides a good measure of the frequency, severity and percentage of various aspects of acidic reflux. It may demonstrate full-column reflux rising to the level of the cricopharyngeus, and so a likelihood of pharyngeal contamination (Figure 3). However recent work using impedance studies has demonstrated that many patients suffer reflux which is not identified on pH monitoring but remains symptomatic and potentially damaging^[17,18]. The pH study does not measure the presence of acid or non-

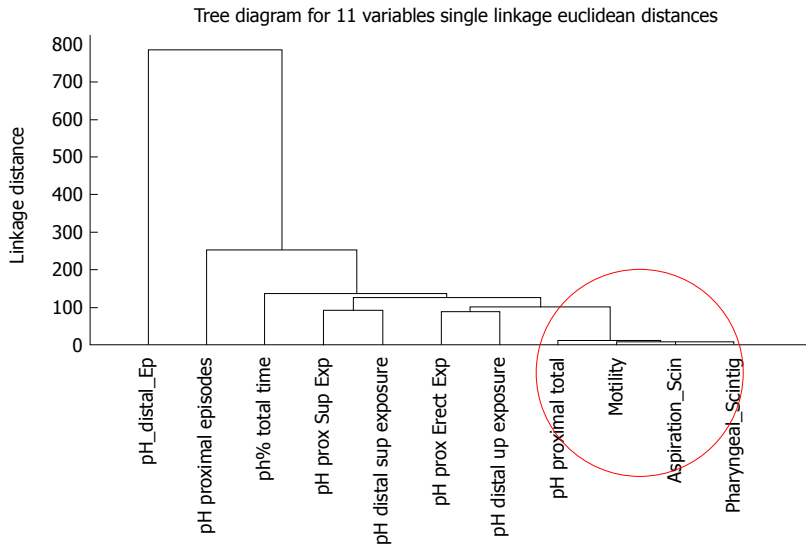


Figure 1 Cluster analysis of variables. There is tight clustering of pH proximal total exposure, Motility, Aspiration scintigraphy and Pharyngeal scintigraphy located at the far right side of the graph.

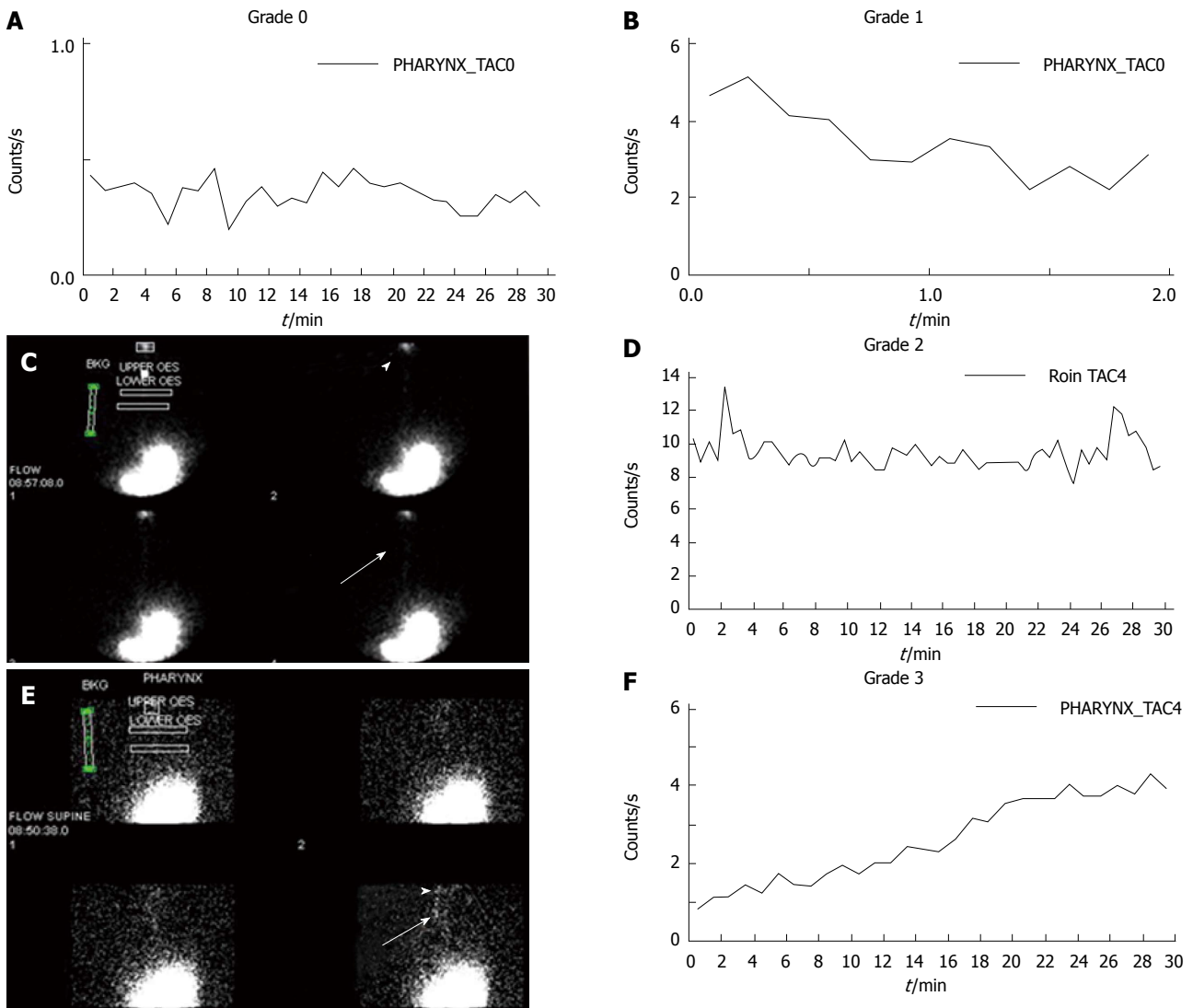


Figure 2 Grading of time-activity curves for the pharynx and upper oesophagus. A: Grade 0 is where there is no significant activity and the curve is similar to the background time-activity curve; B: Grade 1 reflects activity that clears with a falling curve; C, D: Grade 2 is a time activity curve that correlates with activity in the pharynx (arrowhead) and oesophagus (arrow) that fails to clear; E, F: Grade 3 is a rising time-activity curve that indicates progressive gastro-oesophageal reflux (arrowhead and arrow) that indicates rising activity in the pharynx and upper oesophagus respectively.

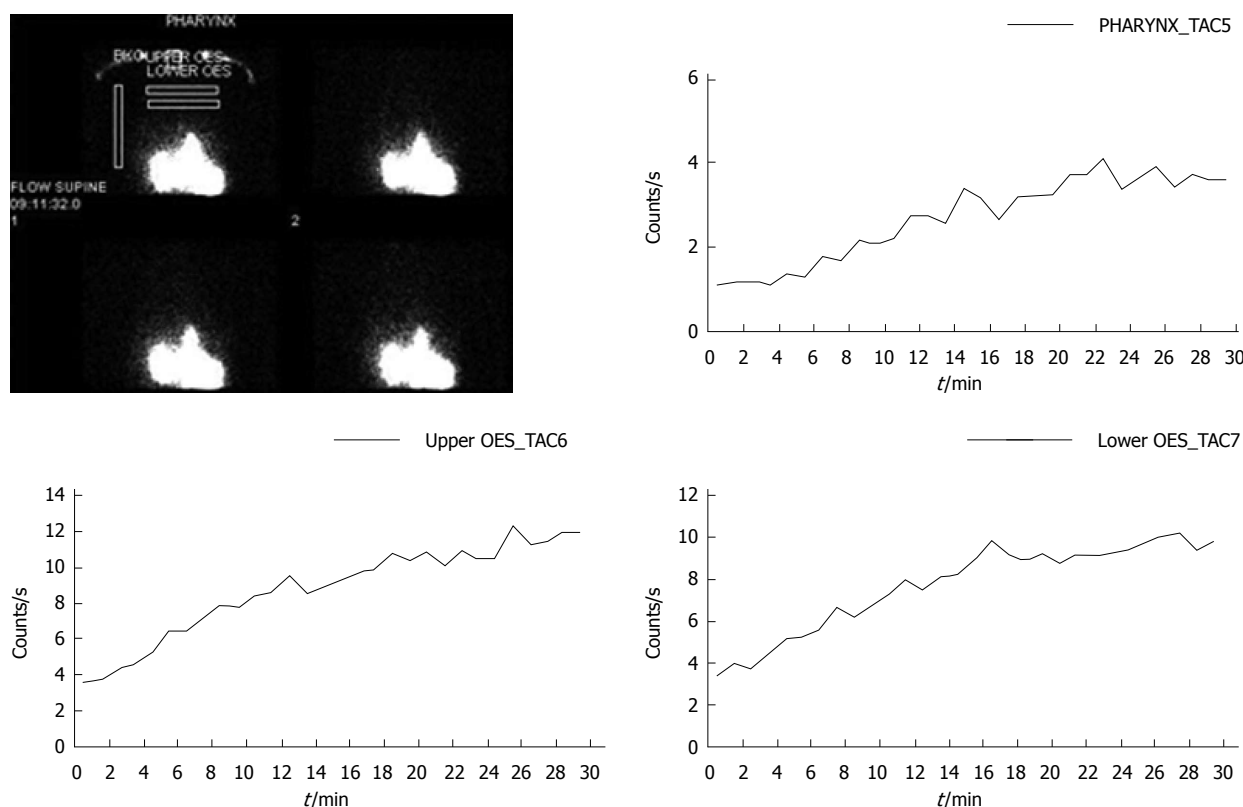


Figure 3 Full column reflux with rising time-activity curves over the pharynx, upper and lower oesophagus. The diagram shows the typical regions of interest from which the data is derived.

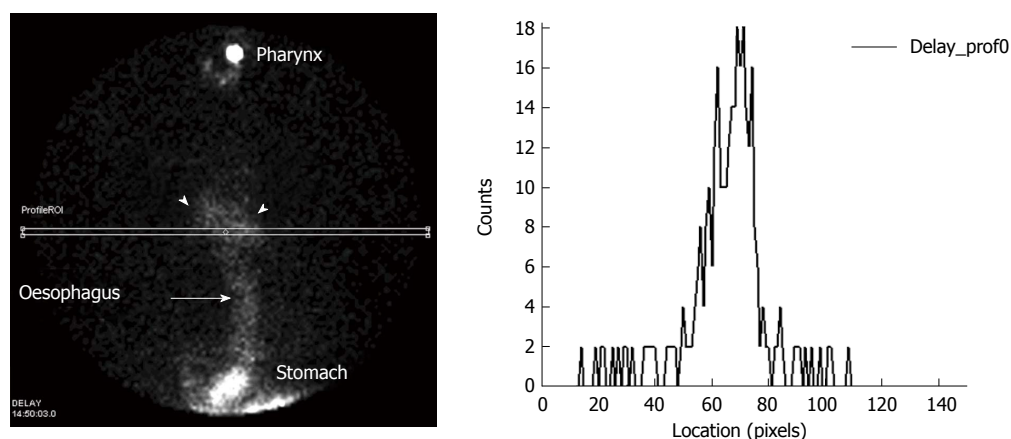


Figure 4 Typical image of the delayed study in which tracer activity is present in the main airways in a patient with lung aspiration.

acid material in the pharynx^[19]. One clear advantage of reflux scintigraphy is that it measures any reflux to the level of the pharynx and the direct occurrence of pulmonary aspiration. It is not dependent upon measuring acid. A rising time-activity curve over the pharynx was demonstrably a good predictor of pulmonary aspiration in this group of patients. It very likely indicates continuing episodes of GERD combined with a failure of normal clearance mechanisms due to impaired motility. A flat pharyngeal time-activity curve may indicate a failure of pharyngeal clearance in the absence of repeated episodes of GERD into the pharynx.

This relationship is reflected in the correlation between the manometric motility studies which measure ineffective oesophageal clearance of acid^[10] and the presence of pulmonary aspiration on scintigraphy.

Upper aero-digestive symptoms and positive pharyngeal scintigraphy were strongly associated when analysed as paired variables on an individual patient basis. Cough, laryngospasm and globus were strongly correlated with positive aspiration scintigraphy. This suggests an aetiological role for the persistence of reflux (acid or weakly acidic/alkaline) fluid in the pharynx and subsequent aspiration into the respiratory

tract (Figure 4). There is evidence that this may directly trigger the cough reflex^[20] or lower the threshold for the cough reflex as indicated by C2 and C5 testing^[21]. The concept of direct damage is supported by the findings in the 25 cases of chronic cough, where 14 cases had aspiration and 23 pharyngeal contamination by scintigraphy. The alternate theory of chronic cough mediated by afferent nerves in the distal oesophagus is also well supported in other studies^[22]. There is no reason to suppose that both mechanisms are not separately operative in individual patients or indeed concurrent as there was no way to identify reflex-mediated patients. However, scintigraphic findings in the current study are not congruent with the concept of a reflex-mediated aetiology as the majority of the patients with cough, laryngospasm and globus had evidence of direct contamination of the pharynx and lungs. Perhaps reflex mediation may be more frequent in a less pathological group. The scintigraphic studies have shown a surprising degree of pulmonary aspiration (50%) in patients with cough. This may reflect the very highly and conservatively selected group of patients with long disease duration, likely to have more severe disease than in other reported series.

The ultimate test of the predictive value of reflux scintigraphic findings has been the symptomatic response to definitive surgical management by laparoscopic fundoplication. Resolution of laryngopharyngeal symptoms were found in 90% of cases. This supports the choice of patients for intervention based on the scintigraphic findings. It is no surprise that there has been a continuation of dysphagia in this patient group with a large percentage of oesophageal dysmotility. It is of course possible that there may have been a strong placebo effect within this patient group, which has been reported to be as high as 85% in chronic cough treated with pharmacological intervention such as opiates. However, opiates also have centralised effects upon cerebral neurotransmitters^[23]. Surgical intervention in this situation is however more likely to have a response rate closer to 35%, which is generally accepted for the placebo effect^[24]. The one patient that had no response to surgery had no definable reflux by pH monitoring or scintigraphy on follow-up. It does demonstrate the complexity of the disease where there may be a mixed pathology of both GERD and primary respiratory disease or a behavioral component. In a large multicentre study of laparoscopic fundoplication for GERD with 5 year follow-up in 1340 patients, symptoms were satisfactorily treated in 93% of patients^[25]. More applicable to this study is a group of 47 patients reported over a six year period having laparoscopic fundoplication for the treatment of chronic cough^[26]. Symptom relief was reported in 30 (64%) with a similar side-effect profile. The use of reflux scintigraphy may have yielded substantially better primary symptom management by improved selection as in our series.

Scintigraphic studies have been utilized to evaluate pulmonary aspiration in infants and children for many years, generally as tracer being instilled in milk which is administered at night with scanning the following morning^[27,28]. These studies have been performed with low radiation exposure, often as low as single chest x-ray examinations and are considered safe and acceptable. Modifications of the technique have been shown to provide good results in the detection of GERD^[29-32] and lung aspiration of refluxate^[33,34]. Results in these series tend to vary with technical differences^[29,31,35,36]. It appears that the volume of liquid in which the tracer is introduced into the stomach is important, as is the framing rate for study acquisition. The optimal volume is reported between 150 and 300 mL^[29,31,32] and framing rates between 15 and 30 s, not 60 s which leads to significant reflux being missed^[37]. Computer modeling and clinical data indicates that as little as 0.1 MBq of activity aspirated into the lung can be detected by the gamma camera^[34]. There is some conflict in the reported reproducibility measures of visual interpretation vs analysis of time-activity curves. In one series, the computerised analysis was significantly better^[38] while in another, visual interpretation was better^[39]. These differences very likely reflect variations in the acquisition parameters. Importantly, reproducibility was good in both studies with kappa values greater than 0.70. Acquisition protocols in the current study were aligned with the technique of Caglar *et al.*^[38]. Two groups have reported good sensitivity and utility of the scintigraphic technique for the detection of laryngeal reflux and aspiration of tracer into the lungs^[35,36]. The study of Bestetti *et al.*^[35] reported 201 patients with symptomatic posterior laryngitis documented by laryngoscopy and who were evaluated after administration of 300 mL of orange juice labeled with 99mTc. GERD was demonstrated by scintigraphy in 134 (67%), of which 78% was proximal and 31 patients were positive on scintigraphy for pulmonary aspiration. These findings are similar to the current report. Symptoms profiles were also similar between the study of Bestetti *et al.*^[35] and our group. Proximal symptoms in our group were tightly correlated with positive pharyngeal contamination. Cough was the only significant variable predicting positive pharyngeal contamination by multivariate analysis. Voice change was the only variable that predicted positive pulmonary aspiration by multivariate analysis. This finding has clinical importance. Our study showed good concordance for oesophageal scintigraphy with the 24 h pH and motility findings on an individual patient basis as paired studies. Other have reported less favourable results for scintigraphy (82% for pH studies vs 33% for scintigraphy)^[31]. This may reflect a different and less severe patient sample. Technical difference may also have contributed to these disparate findings.

This study is relatively small and collected over a

four year period. Such studies are time-consuming and invasive and patient compliance becomes a significant issue. The use of patient questionnaires raises the problem of the degree of placebo effect^[40]. There were a large number of uncontrolled variables in the study and a high degree of patient variability, especially in the pH studies. A valid approach is therefore to consider these variable as dependant paired studies on an individual basis, as none of the variables were held constant. Cluster analysis was a valuable tool to assess a large number of variables and identify fundamental linkages between variables to get a sense of connectedness^[11]. The finding of strong linkages between the motility studies, pH proximal total exposure time, pharyngeal and aspiration scintigraphy is reassuring.

The findings of the current study indicate that reflux scintigraphy utilising the current protocol is a potential screening tool for pharyngeal contamination and lung aspiration if GERD is suspected in patients with cough or other LPR symptoms. This requires further study in a more mixed and less selected group of patients. Subsequent assessment in over 700 patients has shown further utility in predicting response to surgical intervention and more importantly, factors that may predict surgical failure. The technique is simple, reproducible and has a low radiation exposure that is considered acceptable even in a pediatric population. The patient group with long-term LPR symptoms, especially cough and extensive investigation over a long time course can be selected for a high likelihood of symptomatic improvement by laparoscopic fundoplication. The combination of reflux scintigraphy, motility studies and two channel 24 h pH monitoring can increase the likelihood of success to over 90%. Patients with cough should therefore be assessed for pulmonary aspiration.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a common occurrence which may however be asymptomatic and extend to laryngopharyngeal reflux (LPR) and lung aspiration of refluxate. Patients may not have symptoms of GERD but present with typical upper respiratory tract symptoms such as cough, dysphonia and globus. A high clinical index of suspicion is necessary to make the connection. Scintigraphy has the potential to screen for both LPR and lung aspiration.

Research frontiers

Assessing LPR, lung aspiration and predicting the response to surgical therapy with laparoscopic fundoplication is the key to successful therapy. The necessary extension to this is discovering factors that predict failure of surgical therapy. Scintigraphic reflux studies have the potential to perform both tasks.

Innovations and breakthroughs

Impedance studies may prove to be reliable in the assessment of LPR as would salivary pepsin assays. The additive value to pH studies and high resolution manometry may prove to be decisive in the therapy of LPR.

Applications

Scintigraphy may prove to be a good predictor of successful laparoscopic fundoplication in terms of the presence of both LPR and lung aspiration of refluxate. More importantly it may be able to predict failure of surgery, especially

in patients with co-existent gastroparesis. Post-operative assessment may provide important information regarding effectiveness of the fundoplication.

Terminology

Scintigraphy uses the common isotope 99m Technetium which has a half-life of 6 h and a low radiation exposure at the doses being used. It is commonly bound to diethylene triamine pentaacetic acid which is frequently used in renal studies.

Peer-review

The true possible diagnostic role of scintigraphy in LPR might not be so straightforward as hypothesized, and will need more evaluations. Functional components to the symptoms may not be negligible in patients classified as having LPR, thus making more difficult a correct diagnosis, even in case of a positive scintigraphy test.

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Observational Study

Prevalence and factors associated with irritable bowel syndrome among university students in Lebanon: Findings from a cross-sectional study

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Abstract

AIM: To describe the bowel habits and the prevalence of irritable bowel syndrome (IBS) and to investigate the influence of health behavior and social factors on IBS prevalence in university students.

METHODS: This cross-sectional study was conducted at five major universities in Greater Beirut and its suburbs, between February and June 2014. Using a convenience sample, a total of 813 students aged 18 years old and above participated in this study. Participants were asked to complete a comprehensive anonymous questionnaire which detailed characteristics on socio-demographic, health-related, and lifestyle factors, as well as IBS. The ROME III criteria were used as a tool to ascertain IBS. A χ^2 test was used to determine differences between categorical variables; stepwise logistic regression was used to measure the association between IBS and its risk factors.

RESULTS: An overall prevalence of IBS of 20% was recorded among university students. The bivariate analysis showed that females were significantly more likely to report having IBS than males (29.1% vs 18.2%, $P < 0.01$). Those living at the school dormitory or in a private residence (39.5%) were more likely to have IBS than those living with their families (16.3%) ($P < 0.01$). The multivariate analysis showed that those who had a relatively high family income level (US\$ > 2000) were almost 6 times more likely to report having

IBS than their counterparts.

CONCLUSION: This is the first study to describe the nature of IBS among young adults in Lebanon. The prevalence of IBS among university students in our sample was higher than that reported in the West.

Key words: Irritable bowel syndrome; Lebanon; Social factors; Prevalence; ROME III criteria

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Core tip: Irritable bowel syndrome (IBS) is an acknowledged functional gastrointestinal disorder of major public health concern. Little is known about IBS prevalence in Arab countries and specifically among university students, including Lebanon. Therefore, an epidemiological study, the first of its kind, investigating IBS among university students in Lebanon was conducted. The prevalence of IBS reported in this study was relatively high and similar to the estimate found in industrialized countries. The risk of having IBS, after adjusting for confounders was significantly higher among females than males, those aged 22 years or younger, among those who were living in a private house or in the school dormitory on their own, and among subjects with middle to high income levels. Findings of this study have important implications for IBS screening and management, as they highlight the importance of engaging in healthy behaviors to minimize IBS symptoms and enhance quality of life among IBS patients.

Costanian C, Tamim H, Assaad S. Prevalence and factors associated with irritable bowel syndrome among university students in Lebanon: Findings from a cross-sectional study. *World J Gastroenterol* 2015; 21(12): 3628-3635 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3628.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3628>

INTRODUCTION

Irritable bowel syndrome (IBS) is an acknowledged functional gastrointestinal disorder (FGID) that is associated with abdominal pain and bloating, along with other symptoms such as changes in bowel habits^[1]. The worldwide prevalence of IBS ranges from 5.7% to 34%^[2], usually varying significantly between countries according to the diagnostic criteria used^[3]. Various diagnostic tools have been employed for the detection of IBS, including the Manning criteria, Rome I criteria and Rome II criteria^[2]. Currently, the Rome III criteria are the most common method for diagnosing IBS^[4]. Based on the Rome III criteria, the prevalence of IBS has been estimated to range from 10% to 15% in Western countries, whereas that reported in Asian countries ranged from 1% to 10%^[5].

IBS is highly influenced by demographic factors, particularly sex and age. IBS is more prevalent in females than in males and has an onset between late teens to twenties, decreasing with age^[6,7]. Women from developed countries are 2-4 times more likely to have IBS than men^[7,8]. Furthermore, evidence suggests a genetic role in the etiology of IBS, with 33% of patients with IBS reporting a positive family history^[6]. In addition to a genetic predisposition, IBS has also been linked to several dietary habits and to psychological factors such as stress and anxiety^[5,6]. Although IBS is not a grave condition, it does however considerably reduce the quality of life of people afflicted with this syndrome. It interferes with their education, working ability and social life^[9]. Moreover, IBS poses an economic burden to a country's health care system; the annual cost of diagnosing, treating and managing IBS in the United States being between \$1.7 and \$10 billion in direct costs (e.g., from office visits, medications), and up to \$20 billion in indirect costs (e.g., through work absenteeism and reduced productivity)^[10].

Most studies conducted in both developed and developing countries have focused on samples from adolescents and university students. For example, the prevalence of IBS was found to be 5.7% among Korean college students^[11]. A study in China found that medical students had a higher risk of functional bowel disorders than science and engineering students^[12]. Little is known about IBS prevalence in Arab countries and specifically among university students, including in Lebanon, a small middle-income country of the Middle East and North Africa (MENA) region. The sample population chosen is significant because of the lifestyle characteristics that this population experience. Not only are university students relatively restricted in terms of access to a variety of foods but are also exposed to a stress load that accompanies examinations and monetary limitations, thereby potentially exacerbating the onset of IBS symptoms. As a result, an epidemiological study investigating IBS among university students in Lebanon is warranted. Using the Rome III criteria to determine IBS, this study is the first to examine the prevalence and factors associated with IBS among a large sample of university students in Lebanon. The objectives of this study are: (1) to determine the prevalence of IBS among university students in Lebanon; and (2) to investigate the role of socioeconomic and behavioral factors on IBS prevalence in this group of individuals.

MATERIALS AND METHODS

Study design, sample and procedures

This study used a cross-sectional design and was conducted in the Spring semester (February 3-June 2) of the academic year 2013-2014 in Lebanon. According to the Central Administration of Statistics (2011) in

Lebanon, 180850 students were enrolled in higher education institutions in Lebanon during 2009-2010, of which 45.7% were males and 54.3% females. Of the 29 licensed universities in Lebanon, 72530 students (40.2%) were attending the Lebanese University vs 108037 (59.8%) attending private universities^[13]. The study was conducted at five large universities located within the Greater Beirut area, which includes more than 50% of university students in Lebanon. Using a convenience sampling method, participants were recruited from four major private universities, the faculties of which were located in a single campus, and one public university, namely the Lebanese University. Four out of the five private universities approached consented to participate in the study; seven out of nine faculties approached at the Lebanese University, each with its own campus, agreed to participate.

Participants were recruited throughout the semester up until 2 wk prior to the final exam period. Flyers inviting participants to partake in this study were distributed in each of the campuses of the participating universities. To be included in the study, participants had to be between 18-29 years of age and enrolled as undergraduate students in one of the five universities. Students were excluded if they had a history of receiving medication for peptic ulcers or inflammatory bowel disease, such as ulcerative colitis or Crohn's disease. Students who were interested in participating in the study were invited to meet in a large classroom or an auditorium where they were informed about the purpose of the study and were invited to participate in the survey. Participation in the study was voluntary, and did not involve financial or any other compensation. After being screened for inclusion/exclusion criteria, students were then asked to complete a self-administered anonymous questionnaire. During this time, a research assistant was constantly present in the classroom to answer students' questions. The questionnaire required less than 15 minutes to be completed, and included items related to socio-demographic and behavioral characteristics as well as variables pertaining to diagnosis of IBS such as bowel habit and food hypersensitivity. Informed consent of the participants was obtained and all completed questionnaires were anonymous and confidential. Approval to conduct the present study was granted by the administrations of all participating universities. This research was reviewed and approved by the institutional review board at the Lebanese University.

Data collection and measures

Definition of IBS and other covariates: Data on IBS was collected using a validated questionnaire with a reported sensitivity of 65%^[14]: the Rome III criteria. These criteria consist of a standardized self-reported questionnaire, which has been developed by the Rome Foundation Board to identify FGIDs^[15] and is widely

used. The clinical diagnostic criteria defines IBS as recurrent abdominal pain or discomfort for at least 3 d/mo during last 3 mo associated with at least two or more of the following features: (1) improvement after defecation; (2) onset associated with a change in frequency of bowel movement; and/or (3) onset associated with a change in form (appearance) of stools. The diagnosis of IBS can reasonably be made by using the Rome III criteria as long as the patient does not have red flag symptoms like fever, vomiting, rectal bleeding, weight loss, or other findings that may suggest other diagnoses. The classification of IBS subtypes was based on the predominant stool pattern. IBS with constipation (IBS-C) was defined as having hard or lumpy stools at least 25% of the time and loose (mushy) or watery stools in less than 25% of bowel movements. IBS with diarrhea (IBS-D) was defined as having loose (mushy) or watery stools at least 25% of the time and hard stools in less than 25% of bowel movements. Mixed IBS (IBS-M) was defined as having hard or lumpy stool at least 25% of bowel movements and loose (mushy) or watery stool in at least 25% of bowel movements. Un-subtyped or unknown IBS (IBS-U) was defined as an insufficient abnormality of stool consistency to meet the criteria of the other three subtypes.

Socio-demographic, individual and health behavior characteristics were also collected at baseline. These included: sex, age, living condition (at home vs away from home: with a family, in a private house or in the school dormitory), income (US\$ 500-1200; 1300-2000, > 2000), engaging in regular physical activity (yes, no), cigarette smoking (current, not current), and foods that triggered abdominal pain or diarrhea (yes, no; if yes, specify).

Statistical analysis

All eligible questionnaires were coded. Descriptive analyses were performed to determine frequencies of categorical variables and the prevalence of IBS and its subtypes. A χ^2 test was used to determine differences in categorical variables to assess the association between IBS and socio-demographic and behavioral indicators. Multivariate logistic regression was also conducted to predict the independent relationship between each of the socio-demographic and behavioral characteristics as well as family history of IBS, and the risk of IBS. Adjusted odds ratios (ORs) and their 95% confidence intervals are reported. Statistical significance was defined as a *P* value < 0.05, and SPSS 18.0 software package was used for the analyses (IBM Corp., Armonk, NY, United States).

RESULTS

A total of 1000 students were approached to participate in this study; analysis was restricted to 813 participants (325 male, 488 females for whom we

Table 1 Prevalence of irritable bowel syndrome and its subtypes and contributory foodstuffs *n* (%)

Variable	With IBS ¹ (<i>n</i> = 163/813)
IBS	163 (20.0)
IBS subtypes	
IBS constipation	60 (36.8)
IBS diarrhea	25 (15.4)
IBS mixed	73 (44.8)
IBS unknown	5 (3.06)
Foods that trigger hypersensitivity ²	
Yes ³	34 (20.9)
No	129 (79.1)
Beans	8 (23.5)
Milk	4 (11.8)
Sweets	1 (2.94)
Fatty food (beef and fast foods)	4 (11.8)
Coffee	5 (14.7)
Carbonated beverages	1 (2.94)
Spicy foods	5 (14.7)
Fruits (banana, watermelon)	2 (5.88)
Vegetables (tomato)	2 (5.88)
Parsley	2 (5.88)

¹Irritable bowel syndrome (IBS) was defined according to Rome III criteria as recurrent abdominal pain or discomfort for at least 3 d per month during the past 3 mo, associated with two or more of the following features: (1) improvement with defecation; (2) onset associated with a change in frequency of bowel movement; and/or (3) onset associated with a change in form (appearance) of stools; ²In the form of abdominal pain or diarrhea; ³Distribution of specific food categories among IBS cases (*n* = 34) triggering hypersensitivity.

had complete data on the primary outcome, IBS prevalence. No major differences between those with complete information on IBS prevalence and those with incomplete information existed. The mean age of the study sample was 22.7 years (standard deviation, 1.39 years) with a larger proportion of females than males (488 vs 325; 60% vs 40%). Table 1 details IBS prevalence, its subtypes, and the foods that triggered IBS symptoms among the cases. Of 813 participants, 163 fulfilled the Rome III criteria for a diagnosis of IBS, yielding a prevalence of 20.05% (95%CI: 18.95-21.15). Sixty respondents (36.8%) were classified as IBS-C and 25 (15.4%) as IBS-D. The majority (44.8%) of respondents were classified as IBS-M, while the remaining 3% of students fell into the IBS-U subgroup. Almost a quarter (20.9%) of IBS positive cases reported identifying foods that triggered diarrhea or abdominal pain.

Table 2 outlines the characteristics of the overall study population and of students with IBS as well as the results of the regression analysis. These associations remained statistically significant before and after adjustment. The risk of having IBS was significantly higher among females than males (OR = 0.40; 95%CI: 0.26-0.61), those aged ≤ 22 years (OR = 0.53; 95%CI: 0.35-0.79), and those who were living in a private house or school dormitory on their own compared to those who were living with their family (OR = 2.84; 95%CI: 1.94-4.16). The majority of participants had a relatively low to middle income level,

with IBS being significantly higher among subjects with higher income levels (OR = 5.72; 95%CI: 3.36-9.71). In contrast, students who performed regular physical activity had a 53% reduction in IBS prevalence compared to those who did not perform regular physical activity (95%CI: 0.35-0.79). Over 50% of students who reported having IBS were in the Faculty of Medical Sciences and had majors such as medicine, pharmacy, and dentistry, although this was not statistically significant. Furthermore, the majority of respondents with IBS did not report a family history of IBS.

DISCUSSION

The prevalence of IBS reported in this study was relatively high at 20%. This is similar to the estimate found in industrialized countries (2%-19%)^[5]. Almost a quarter of IBS cases reported identifying foods that triggered diarrhea or abdominal pain. The risk of having IBS was significantly higher among females than males, those aged 22 years or younger, and those who were living in a private house or school dormitory on their own, and among subjects with middle to high income levels, after adjustment for the effect of other covariates. This study also suggested that engaging in regular physical activity, was a protective factor for IBS. A positive family history of IBS, albeit non-significant, had a protective effect against having IBS.

While the 20% prevalence rate of IBS among university students in Lebanon is relatively higher than that found in Western countries, it is similar to that reported by other countries in the region. IBS affects about 10%-15% of adults in North America^[14]. Consistent with the prevalence rate obtained in this study, a study conducted among medical students at the University of Western Ontario in Canada, found that the prevalence of IBS among preclinical and clerkship students was 19.1% and 22.0%, respectively^[16]. However, in Asian countries a highly variable range of IBS prevalence has been observed (2.3%-34%)^[11]. Studies in the MENA region are scarce and mostly restricted to special groups of students or among patients in hospital settings^[3,17,18]. A study conducted among secondary school male students in Al-Jouf Province in Saudi Arabia showed a 9.2% prevalence of IBS^[19]. Another study conducted among medical students and interns at the King Abdulaziz University in Jeddah, found a prevalence of 31.8% according to the Rome III criteria^[18], while Naeem and colleagues^[20], also using the Rome III criteria, reported a 28.3% prevalence of IBS among medical students in Karachi, Pakistan. Abdulmajeed *et al*^[3] found a prevalence of 34.2% using the Rome II criteria in a study of 117 primary health care center attendees at Suez governorate in Egypt.

Less than a quarter of positive IBS cases reported having food hypersensitivity by identifying foods that triggered diarrhea or abdominal pain. Reported

Table 2 Distribution of baseline socio-demographic and behavioral characteristics and factors associated with irritable bowel syndrome prevalence *n* (%)

Variable	Total (<i>n</i> = 813)	With IBS (<i>n</i> = 163)	<i>P</i> value	Unadjusted		Adjusted ¹	
				OR	95%CI	OR	95%CI
Sex							
Female	488 (60.0)	122 (25.0)	< 0.001	1.00		1.00	
Male	325 (40.0)	41 (12.6)		0.43 ^a	0.29-0.64	0.40 ^a	0.26-0.61
Age							
≤ 22 yr	539 (66.3)	118 (21.9)	0.039	1.00		1.00	
> 22 yr	274 (33.7)	45 (16.4)		0.7	0.48-1.02	0.53 ^a	0.35-0.79
Faculty							
Sciences and humanities	113 (13.9)	22 (19.5)	0.975	1.00		1.00	
Medical sciences ²	431 (53.0)	89 (20.6)		0.98	0.53-1.85	0.80	0.45-1.42
Engineering	140 (17.2)	27 (19.3)		1.07	0.64-1.81	1.06	0.53-2.11
Business and economics	129 (15.9)	25 (19.4)		0.99	0.52-1.80	0.94	0.47-1.89
Living condition							
With family	517 (63.6)	75 (14.5)	< 0.001	1.00		1.00	
School dormitory or private house	296 (36.4)	88 (29.7)		2.49 ^a	1.76-3.54	2.84 ^a	1.94-4.16
Family income (US\$)							
500-1200	241 (29.6)	28 (11.6)	< 0.001	1.00		1.00	
1300-2000	369 (45.4)	64 (17.3)		1.60	0.99-2.57	1.86 ^a	1.13-3.06
> 2000	203 (25.0)	71 (35.0)		4.09 ^a	2.51-6.68	5.72 ^a	3.36-9.71
Cigarette smoking							
Not current	716 (88.1)	144 (20.1)	0.514	1.00		1.00	
Current	97 (11.9)	19 (19.6)		0.97	0.56-1.60	0.95	0.43-1.72
Regular physical activity							
No	474 (58.3)	113 (23.8)	0.001	1.00		1.00	
Yes	339 (41.7)	50 (14.7)		0.55 ^a	0.38-0.80	0.53 ^a	0.35-0.79
Family history of IBS							
No	754 (92.7)	154 (20.4)	0.401	1.00		1.00	
Yes	59 (7.3)	9 (15.3)		0.70	0.34-1.46	0.49	0.22-1.08

¹Adjusted for all variables listed in the table; ²Includes medicine, dentistry, pharmacy, public health, medical lab technology, and physiotherapy. ^a*P* < 0.05 vs control. OR: Odds ratio; CI: Confidence interval.

perceived triggers included carbohydrates and fatty foods, together with caffeine, alcohol and spices. To date, few studies have examined the dietary intakes of IBS patients to identify dietary changes or potential nutrient deficiencies^[21]. This was consistent with findings from a cross-sectional study across a large sample of Iranian adults which found that women with high consumption of spicy foods had a 2-fold increased risk of developing IBS compared with women who reported not consuming any spicy foods^[22]. On the other hand, the frequency of meals per day and frequency of flour intake were not associated with IBS, similar to findings reported by Ibrahim *et al*^[18] among medical students in King Abdulaziz University in Jeddah. The results above underline the need for further studies to characterize potential relationships between diet-related practices and the risk of FGID, in order to design appropriate and effective diet-based interventions.

We found that the risk of having IBS was significantly greater among females than males. This finding is in concordance with the established association between female sex and IBS^[23]. In fact, most studies conducted in Western countries showed that IBS affected women more than men^[24]. A systematic review pooling the results of 80 studies showed that the prevalence of IBS was significantly higher for women than men (overall OR = 1.67); these

included 55 studies that mostly reported a positive association between IBS and female sex^[25]. Similar results were also found in countries of the MENA region where a systematic review on the prevalence and risk factors of IBS in the Republic of Iran showed that more than half of the reviewed studies showed that the prevalence of IBS was significantly correlated with female sex^[26]. However, the reason behind the sex difference in IBS prevalence remains uncertain. A possible explanation for this might lie in the differences in socio-cultural features such as health care seeking behavior between men and women or it may be due to actual biological differences. For example, although gastrointestinal symptom changes related to the menstrual cycle are common in women in general, those who have IBS are significantly more likely to report an exacerbation of bowel symptoms during menses^[27].

Interestingly, living arrangements or conditions were associated with IBS prevalence in our sample. The proportion of students with IBS who were living away from home was higher than that of students living at home. A possible explanation might lie in the fact that life away from home may have an influence on lifestyle behaviors including irregular dietary habits and poor stress management, thereby influencing the manifestation of IBS symptoms. This possibility was demonstrated by Mansour-Ghanaei *et al*^[28] who

reported that Iranian students living at a distance from their families had significantly higher rates of IBS compared to others. The effect of stress as a major moderator in IBS development has been previously explored and established^[29], yet the exact mechanism of how psychological stress induces abdominal symptoms has not been elucidated, despite many studies reporting a bidirectional relationship between the central nervous system and the digestive tract^[30]. Since stress and other psychological factors are associated with IBS, living away from home may act as a potential stressor, affecting the onset and severity of IBS among vulnerable students.

The role of a subject's socioeconomic status (SES) may also play a possible role in IBS development. Studies have demonstrated a link between affluent childhood SES and adult Manning criteria for IBS^[31,32]. An association between a higher socioeconomic environment and IBS was also noted in our sample. A significant proportion of students who lived away from home, in a private apartment or house, had middle to high family income. Moreover, *post hoc* analysis showed that over 50% of IBS positive cases living away from home had middle to high family income. A possible explanation for this association lies in the "hygiene hypothesis" proposed by Gwee^[33]. Children from a high social class are less likely to live in highly crowded environments and as a result, are less exposed to enteric pathogens at an early stage of life. These pathogens result in the development of immune tolerance by protecting against the development of post-infectious IBS through increased exposure to intestinal organisms leading to lower risk of adult IBS^[33].

Regarding behavioral risk factors, the results of the present study showed that IBS prevalence was higher among students who did not engage in physical exercise. These results are similar to those obtained by Kim *et al.*^[34] who reported a higher prevalence of IBS among Korean university students who did not exercise. Similarly, Dong *et al.*^[11] found that low exercise levels indicated a high risk of IBS among Chinese university students. Increased physical activity has been shown to improve IBS symptoms whereby physically active IBS patients have less symptom exacerbation compared with physically inactive patients^[35]. Physical activity also has a well-known role in stress management; therefore, it is possible that patients with IBS who did not exercise might have been less able to cope with a stressful factor in their lives, be it personal or socioeconomic, in addition to the university experience being itself an additional stressor, thereby exacerbating IBS symptoms.

Strengths

Despite the relatively high prevalence of IBS in Asia in general and in the Middle East in particular, few studies in the region have examined the prevalence in this

area. Therefore, this study is unique in its nature, as it is the first study in Lebanon to analyze the predictors of IBS among university students at the multivariate level. The sample size of the study was heterogeneous (private and public universities), and not restricted to one university, reflecting the socioeconomic diversity of all university students in Lebanon. Moreover, the ROME III criteria have been shown to be a reliable and valid tool to diagnose and obtain estimates of FGIDs based on symptoms for various gastrointestinal conditions^[15].

Limitations

Findings from this study should be interpreted with consideration of some limitations. Data in the study were self-reported with no verification performed, incurring a possibility of information bias. Also, misclassification bias of the dependent variable might have occurred because of the use of self-completed questionnaires. However, because the questionnaires were anonymous, the likelihood of misclassification bias was minimized. Selection bias may have been possible since the sample was not random and may not be representative of the whole university student population in Lebanon, and so our results cannot be generalized to this target population. Moreover, given the exploratory nature of the study, certain confounders were not controlled for, possibly introducing some confounding bias. Finally, due to the cross-sectional nature of our data, we cannot infer causality from these findings.

In conclusion, IBS presents a major public health concern worldwide due to its negative impact on health-related quality of life and high health care expenditure. This is the first study to describe the nature of IBS among young adults in Lebanon. Our study found that the prevalence of IBS in a large population of Lebanese students was higher than rates reported in the West. This study showed that proportionately more women suffered from IBS than men and that social as well as health behavioral factors have significant influences on the presence and progression of IBS. As a result, multifaceted interventions should be considered when aiming to reduce symptoms of IBS such as dietary education, and encouragement to change lifestyles in order to control stress.

The findings of this study also have important implications for IBS screening and management, as they highlight the importance of engaging in healthy behaviors such as physical activity to minimize IBS symptoms and enhance quality of life. It is worthwhile to note that Lebanon has continuously been facing insecurity and political conflict. These factors are not present in countries where other studies have been conducted and such contextual factors are expected to have an adverse effect on physical and mental health and so any intervention that targets Lebanese youth should be tailored to suit the environment and barriers

found in this country. More studies are needed to fully explore the factors related to symptoms of FGIDs in Lebanon. Future studies that include objective measurement of dietary factors and habits, exercise and psychological factors are needed to add to the understanding of the scope and dimensions of IBS in this population.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a significant public health issue and is considered to be prevalent in the general population, but no data on bowel habits and IBS among young adults exists in Lebanon.

Research frontiers

The objective of this study was to describe the lifestyle and other factors associated with IBS prevalence among university students in Lebanon.

Innovations and breakthroughs

This is the first published paper describing the nature of IBS among young adults in Lebanon. The prevalence of IBS among university students in our sample was higher than that reported in the West. This study shows that proportionately more women suffered from IBS than men and that lifestyle factors were significantly associated with IBS.

Applications

Findings from this study have important implications for programs intended to improve academic performance, stress management and quality of life among students suffering from IBS.

Peer-review

The manuscript investigates the nature of IBS among young adults in Lebanon. The topic of the paper is interesting and important.

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Prospective Study

Helicobacter pylori and serum kynurenine-tryptophan ratio in patients with colorectal cancer

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Author contributions: Engin AB performed the majority of the experiments, designed the study and wrote the manuscript; Karahalil B and Karakaya AE edited the manuscript; and Engin A co-ordinated and provided the collection of human material, designed the study and edited the manuscript.

Ethics approval: The study was approved by Gazi University, Local Ethics Committee.

Informed consent: All participants' rights were protected and informed consents were obtained according to the Helsinki Declaration.

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Data sharing: Participants gave informed consent for data sharing.

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Abstract

AIM: To evaluate how *Helicobacter pylori* (*H. pylori*) is able to evade the immune response and whether it enhances systemic immune tolerance against colorectal cancer.

METHODS: This prospective randomized study involved 97 consecutive colorectal cancer patients and 108 cancer-free patients with extra-digestive diseases. Colorectal cancer and cancer-free patients were assigned into subgroups according to *H. pylori* IgG seropositivity. Exposure to *H. pylori* was determined by IgG seropositivity which was detected by enzyme linked immunoassay (ELISA). Serum neopterin levels were measured by ELISA. Serum tryptophan, kynurenine, and urinary bipterin concentrations were measured by high performance liquid chromatography. Serum nitrite levels were detected spectrophotometrically. Serum indoleamine 2,3-dioxygenase activity was estimated by the kynurenine to tryptophan ratio and by assessing the correlation between serum neopterin concentrations and the kynurenine to tryptophan ratio. The frequencies of increased serum kynurenine to tryptophan ratio of *H. pylori* seronegative and seropositive colorectal cancer subgroups were estimated by comparing them with the average kynurenine to tryptophan ratio of *H. pylori* seronegative tumor-free patients.

RESULTS: Compared with respective controls, in both *H. pylori* seronegative and seropositive colorectal cancer patients, while serum tryptophan levels were decreased (controls vs patients; seronegative: $20.37 \pm 0.89 \mu\text{mol/L}$ vs $15.71 \pm 1.16 \mu\text{mol/L}$, $P < 0.05$; seropositive: $20.71 \pm 0.81 \mu\text{mol/L}$ vs $14.97 \pm 0.79 \mu\text{mol/L}$, $P < 0.01$) the kynurenine to tryptophan ratio was significantly increased (controls vs patients; seronegative: 52.85

$\pm 11.85 \mu\text{mol}/\text{mmol}$ vs $78.91 \pm 8.68 \mu\text{mol}/\text{mmol}$, $P < 0.01$, seropositive: $47.31 \pm 5.93 \mu\text{mol}/\text{mmol}$ vs $109.65 \pm 11.50 \mu\text{mol}/\text{mmol}$, $P < 0.01$). Neopterin concentrations in cancer patients were significantly elevated compared with controls ($P < 0.05$). There was a significant correlation between serum neopterin levels and kynurenine/tryptophan in control and colorectal cancer patients groups ($r_s = 0.494$, $P = 0.0001$ and $r_s = 0.293$, $P = 0.004$, respectively). Serum nitrite levels of *H. pylori* seropositive cancer cases were significantly decreased compared with seropositive controls (controls vs patients; $26.04 \pm 2.39 \mu\text{mol}/\text{L}$ vs $20.41 \pm 1.48 \mu\text{mol}/\text{L}$, $P < 0.05$). The decrease in the nitrite levels of *H. pylori* seropositive cancer patients may be attributed to excessive formation of peroxynitrite and other reactive nitrogen species.

CONCLUSION: A significantly high kynurenine/tryptophan suggested that *H. pylori* may support the immune tolerance leading to cancer development, even without an apparent upper gastrointestinal tract disease.

Key words: Colorectal cancer; Kynurenine/tryptophan; Immune tolerance; *Helicobacter pylori*; Oxidative stress

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Core tip: Persistent inflammation of the stomach induced by *Helicobacter pylori* (*H. pylori*) can have consequences on the rest of the body. Despite the vigorous innate and adaptive immune response against the bacterium, *H. pylori* escape and evade host responses by a variety of mechanisms. Low tryptophan levels and increased concentrations of its degradation product, kynurenine, may be directly involved in diminished T-cell responsiveness to antigenic stimulation in cancer. *H. pylori* seropositive colorectal cancer patients with significantly higher kynurenine/tryptophan and reduced nitric oxide suggested that *H. pylori* might support immune tolerance leading to cancer development, even in patients without an apparent upper gastrointestinal tract disease.

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INTRODUCTION

Persistent inflammation of the stomach induced by *Helicobacter pylori* (*H. pylori*) can have consequences on the rest of the body. Recent studies showed that the immunological response against *H. pylori* is not only

a locally but also a systemically evoked phenomena in the host. Particularly in the last few years, many studies have been performed on the role of *H. pylori* in the pathogenesis of extra-gastric diseases^[1]. It is now known that exposure to *H. pylori* and subsequent seropositivity is associated with an increased number of cardiovascular, respiratory, extra-gastrointestinal digestive, neurological and miscellaneous autoimmune disorders^[2].

It has previously been shown that the risk of colon adenomas is increased in *H. pylori*-infected subjects. Two recent studies from Japan, based on a large number of patients, added important evidence for the association between *H. pylori* infection and colorectal neoplasia. Previously, Fujimori *et al.*^[3] showed a positive relationship between *H. pylori* infection and the risk of adenoma and carcinoma, especially in women [odds ratio (OR); 1.68 and 2.09, respectively]. Later, Mizuno *et al.*^[4] found that *H. pylori* infection was associated with the presence of colon adenomatous polyps. A very significant increase in the incidence of adenomas was observed in the seropositive group compared with seronegative controls (44.3% vs 18.9%, $P < 0.0001$).

In the evaluation of the relationship between *H. pylori* and risk of colorectal cancer, the estimated OR showed a small increase in the risk of colorectal cancer development because of *H. pylori* infection. Recently, in one of two different meta-analyses, an OR of 1.49 (95%CI: 1.17-1.91) was found for the association between *H. pylori* infection and colorectal cancer. In another study, serological investigation demonstrated an OR of 1.56 (95%CI: 1.14-2.14) for the association between immunoglobulin G (IgG) antibody and colorectal cancer risk^[5,6]. Likewise, in our previous study, we found a 2.2-fold increase in the risk of colorectal carcinoma in patients with *H. pylori* IgG seropositivity^[7]. Very recently, a significant correlation was found by Popović and colleagues between *H. pylori* seropositivity and colon cancer ($P = 0.002$) in a series of 142 patients^[8].

Although the human host mounts a vigorous innate and adaptive immune response against the bacterium, *H. pylori* can escape and evade host responses by a variety of mechanisms that lead to persistent colonization and chronic active inflammation^[9]. Surprisingly, most people infected with *H. pylori* are asymptomatic, which suggests that additional factors are necessary for the development of *H. pylori*-associated diseases^[10].

Thomas and Stocker proposed a sequential defense mechanism for the immune response, in which indoleamine 2,3-dioxygenase (IDO) activity is the first-line of defense against invading cells^[11]. Interferon-gamma (IFN- γ)-induced IDO activity mediates an antimicrobial effect. During the first phase of infection, IDO-mediated tryptophan (Trp) depletion is predominantly antimicrobial whereas in the further stage, it is an inhibitor of T-cell growth^[12].

Despite the phenomenon of immune activation against cancer cells, low Trp levels and increased concentrations of its degradation products may be directly involved in diminished T-cell responsiveness to antigenic stimulation in cancer patients^[13]. This new mechanism proposed for tumoral immune resistance involves the expression of IDO by tumor cells. It rapidly degrades Trp, and resultant Trp depletion causes a strong inhibitory effect on the development of immune responses^[14].

These evidences raise the question of how *H. pylori* is consistently able to evade these cellular and humoral immune responses. Furthermore, it is not known whether *H. pylori* enhance systemic immune tolerance against colorectal cancer or not. Regarding the mentioned assumptions, the present study was designed to determine the effects of the serum kynurenine (Kyn)/Trp, serum Trp, Kyn and neopterin levels, which are sustained in persisting *H. pylori* seropositivity in colorectal cancer patients.

MATERIALS AND METHODS

This prospective randomized study involved 97 consecutive colorectal cancer patients; 61.7 ± 1.3 (mean \pm SE) years of age, and body mass index (BMI) of 25.5 ± 0.4 kg/m², and 108 cancer-free patients with extra-digestive diseases; 55.5 ± 1.3 years of age, and BMI of 27.1 ± 0.5 kg/m², referred to Gazi University, Faculty of Medicine, Department of General Surgery for surgical evaluation. Diagnosis was made by colonoscopy and histological examination of tumor biopsies in all colorectal cancer patients, and stratified according to the TNM classification of the American Joint Committee on Cancer Staging. From laboratory findings and clinical staging, the primary disease of all cancer patients was found to be suitable for surgical intervention. Subsequent confirmation of the preoperative diagnosis was made by routine histopathological examination of postoperative specimens regarding the presence of lymphatic invasion, lymph node involvement, peritumoral lymphoid cell infiltration, and tumor grade. However, these histological findings were ignored in our series of patients because of the high seroprevalence of *H. pylori* in the general population and prevalent asymptomatic infection makes the interpretation of the definite role of *H. pylori* difficult. Therefore, cancer patients and cancer-free cases were divided simply into two subgroups, based on the presence or absence of *H. pylori* IgG seropositivity. Exposure to *H. pylori* in each patient was determined by an IgG seropositivity test (ELISA) according to the manufacturer's instructions (*H. pylori* IgG ELISA, Demeditec, Germany). Considering the ELISA kit manual, individuals with an *H. pylori* IgG titer below 8 U/mL were accepted as *H. pylori* seronegative, while a value above 12 U/mL was interpreted as *H. pylori*

seropositive. The patients whose titers were between 8 and 12 U/mL appeared in the grey zone and were not included in the study. The 13C-urea breath test (UBT) is an accurate, non-invasive test to diagnose gastric viable *H. pylori* colonization in adults and it is also used to monitor the outcome of eradication therapy in patients^[15]. However, findings suggest that infections with *H. pylori* may have a long-lasting impact on the cell-mediated immune system even after viable bacteria are eradicated. Therefore, we did not perform UBT in our series of patients but determined *H. pylori* IgG seropositivity in our study. There were 37 *H. pylori* IgG seronegative and 71 *H. pylori* IgG seropositive control patients, while 19 of the colorectal cancer patients were *H. pylori* IgG seronegative and 78 were *H. pylori* IgG seropositive.

Cancer-free individuals were selected from the patient population admitted to the hospital for herniorraphy, hemorrhoidectomy or breast biopsies. These patients had undergone diagnostic endoscopic evaluation whenever their complaints were suggestive of digestive diseases. Patients who did not have any pathological finding in either the upper or lower gastrointestinal system were included in the cancer-free control group. No patient had cardio-pulmonary or metabolic risks that could be an obstacle for surgery.

The exclusion criteria were immune system disorders, not able to receive surgical intervention or treatment with neoadjuvant chemotherapy because of late stage carcinoma, or having malnutrition, autoimmune diseases, systemic inflammatory response syndrome, chronic granulomatosis, collagen tissue or neurodegenerative diseases. None of the patients who received traditional triple eradication therapy for *H. pylori* infection within the last two years were included in the study groups.

All participants' rights were protected and informed consents were obtained according to the Helsinki Declaration. Gazi University, Local Ethics Committee approved the study protocol.

Peripheral venous blood samples from each individual was collected and used for serum separation. Urine specimens were collected coincidentally. All samples were obtained in the early morning, and kept from direct light at -20 °C until assay.

Bioppterin and creatinine levels in urine were analyzed by high performance liquid chromatography (HPLC), as previously described^[16]. Serum neopterin concentrations were determined according to the manufacturer's instructions by a commercially available enzyme immunoassay kit (ELISA, Tani Medical Laboratories, Ankara, Turkey). The optical density was measured at 450 nm. Trp and Kyn concentrations in serum were determined simultaneously by reversed-phase HPLC. In order to estimate Trp degradation, the Kyn to Trp ratio (Kyn/Trp) was calculated by dividing Kyn concentrations (μ mol/L) by Trp concentrations (mmol/L)^[17]. The frequency of increased serum Kyn/Trp of *H. pylori* seronegative and seropositive colorectal

Table 1 Comparison of neopterin, tryptophan, kynurenine, nitrite, biopterin levels (mean \pm SE) in controls without malignancy and in gastric cancer patients

	Control group (<i>n</i> = 108)	Colorectal cancer group (<i>n</i> = 97)	<i>P</i> value
Serum neopterin (nmol/L)	7.68 \pm 0.56	21.18 \pm 2.45	0.021 ^a
Tryptophan (nmol/L)	20.6 \pm 0.61	15.38 \pm 0.64	0.000 ^a
Kynurenine (nmol/L)	0.89 \pm 0.09	1.24 \pm 0.09	0.000 ^a
Kynurenine/tryptophan (μ mol/mmol)	49.10 \pm 5.52	99.93 \pm 8.70	0.000 ^a
Nitrite (μ mol/L)	24.60 \pm 1.69	23.05 \pm 1.74	0.107
Urinary biopterin/ creatinine (μ mol/mol)	121.65 \pm 7.79	122.70 \pm 7.69	0.926

^a*P* < 0.05, control vs cancer patients.**Table 2** Comparison of neopterin, tryptophan, kynurenine, nitrite, biopterin levels (mean \pm SE) in *Helicobacter pylori* seronegative controls and *Helicobacter pylori* seronegative colorectal cancer patients

	<i>H. pylori</i> seronegative control group (<i>n</i> = 37)	<i>H. pylori</i> seronegative colorectal cancer group (<i>n</i> = 19)	<i>P</i> value
Serum neopterin (nmol/L)	8.10 \pm 1.11	20.89 \pm 6.27	0.010 ^a
Tryptophan (nmol/L)	20.37 \pm 0.89	15.71 \pm 1.16	0.003 ^a
Kynurenine (nmol/L)	0.89 \pm 0.12	1.22 \pm 0.18	0.007 ^a
Kynurenine/tryptophan (mmol/mmol)	52.85 \pm 11.85	78.91 \pm 8.68	0.000 ^a
Nitrite (mmol/L)	21.86 \pm 1.81	25.51 \pm 4.34	0.938
Urinary biopterin/ creatinine (mmol/mol)	116.20 \pm 12.87	119.1 \pm 11.6	0.573

^a*P* < 0.05, *Helicobacter pylori* (*H. pylori*) seronegative control vs *H. pylori* seronegative cancer patients.

cancer groups was estimated by comparing each of them with the mean of seronegative cancer-free controls. Serum nitrite concentrations were measured by the Griess method^[18]. Serum nitrate measurements were not included in this study, because nitrates are not only released as the final products of nitric oxide (NO) oxidation via nitrites, but could also be produced from peroxynitrites formed during the reaction of NO with the free oxygen radicals. A conclusive profile of NO concentration is reflected by serum nitrite level^[19]. Measurement of serum creatinine levels was performed by an auto analyzer.

Statistical analysis

Data were analyzed using SPSS, version 13.0 (SPSS Inc., Chicago, IL, United States). All results are expressed as mean \pm SE. After checking the data with the Kolmogorov-Smirnov test, non-parametric data of two independent groups were compared with the Mann-Whitney *U* test and *P* < 0.05 was considered statistically significant. Correlations were assessed using the Spearman rank test.

Table 3 Comparison of neopterin, tryptophan, kynurenine, nitrite, biopterin levels (mean \pm SE) in *Helicobacter pylori* seropositive controls and *Helicobacter pylori* seropositive colorectal cancer patients

	<i>H. pylori</i> seropositive control group (<i>n</i> = 71)	<i>H. pylori</i> seropositive colorectal cancer group (<i>n</i> = 78)	<i>P</i> value
Serum neopterin (nmol/L)	7.44 \pm 0.61	21.8 \pm 3.05	0.0001 ^a
Tryptophan (nmol/L)	20.71 \pm 0.81	14.97 \pm 0.79	0.0001 ^a
Kynurenine (nmol/L)	0.89 \pm 0.13	1.28 \pm 0.11	0.0001 ^a
Kynurenine/tryptophan (μ mol/mmol)	47.31 \pm 5.93	109.65 \pm 11.50	0.0001 ^a
Nitrite (μ mol/L)	26.04 \pm 2.39	20.41 \pm 1.48	0.017 ^a
Urinary biopterin/ creatinine (μ mol/mol)	124.65 \pm 9.84	120.69 \pm 9.98	0.607

^a*P* < 0.05, *Helicobacter pylori* (*H. pylori*) seropositive control vs *H. pylori* seropositive cancer patients.

RESULTS

Mean serum neopterin levels of the cancer patients were significantly lower than in the non-tumor group (Table 1). In the evaluation of subgroups, there was a significant increase in serum neopterin in both *H. pylori* seronegative (Table 2) and seropositive (Table 3) cancer patients (*u* = 202, *P* = 0.01 and *u* = 1265, *P* = 0.0001, respectively) compared with their matched controls. However, the highly significant increase in serum neopterin levels in *H. pylori* seropositive cancer patients suggested that *H. pylori* seropositivity enhanced the immune response of macrophages against colorectal cancer. Considering all colorectal cancer patients, although the reduction in serum nitrite concentrations was not significant (*u* = 5538.5, *P* = 0.107) (Table 1), serum nitrite levels of *H. pylori* seropositive cancer patients were significantly lower compared with *H. pylori* seropositive cancer-free matched controls (2110, *P* = 0.01) (Table 3). Serum nitrite levels did not differ between the *H. pylori* seronegative cancer group and their matched controls (*u* = 347, *P* = 0.938) (Table 2). These findings may be consistent with the evidence that the released reactive oxygen and nitrogen species are produced by the phagocytic leukocytes which are recruited to the colorectal tumor site and by *H. pylori* itself. However, it was not investigated whether *H. pylori* colonization is still positive or not in 78 seropositive cases with colorectal cancer. Unchanged urinary biopterin, oxidized product of tetrahydrobiopterine (H4-bip) in *H. pylori* seropositive colorectal cancer patients (1385.5, *P* = 0.607) (Table 3) showed that despite the increased consumption of NO, supportive NO synthesis in these patients was not evident.

Trp concentrations in cancer patients were significantly lower than that of cancer-free controls (*u* = 2876, *P* = 0.0001) (Table 1). As a result of increased

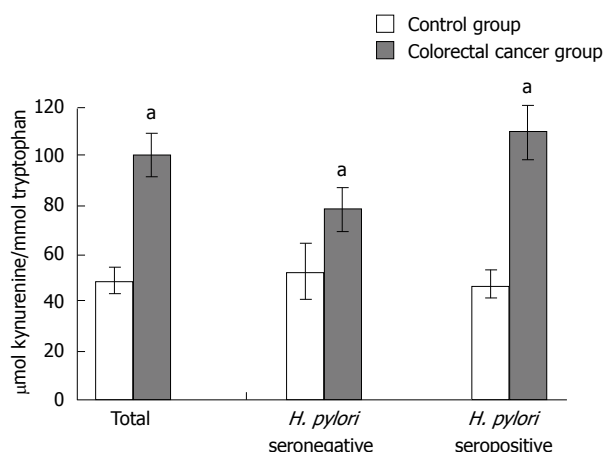


Figure 1 Comparison between kynurenine to tryptophan ratios in controls and colorectal cancer patients, in *Helicobacter pylori* seronegative or seropositive groups. ^a $P < 0.05$, controls vs colorectal cancer patients, *Helicobacter pylori* (*H. pylori*) seronegative controls vs *H. pylori* seronegative colorectal cancer patients, *H. pylori* seropositive controls vs *H. pylori* seropositive colorectal cancer patients, statistically significant.

IDO activity, levels of Kyn, the toxic product of the Trp degradation pathway, significantly increased in colorectal cancer cases ($u = 2931$, $P = 0.0001$). Thus, colorectal cancer patients showed a highly significant rise in Kyn/Trp ($u = 2272$, $P = 0.0001$) (Table 1, Figure 1). To elucidate the contribution of *H. pylori* seropositivity to the frequency of increased Kyn/Trp, the data of *H. pylori* seronegative or seropositive subgroups were evaluated. *H. pylori* seronegative or seropositive colorectal cancer patients had significantly higher Kyn/Trp compared with their matched cancer-free controls. Although no statistical difference was found between IDO activities of *H. pylori* seronegative and seropositive cancer patients, the frequency of increased Kyn/Trp was estimated as 46% and 57% for seronegative and seropositive subgroups, respectively. This means that *H. pylori* seropositivity might add 11% to the frequency of increased Kyn/Trp in the colorectal cancer group and enhance immune tolerance against cancer cells. There was a significant correlation between the serum neopterin levels and Kyn/Trp in control and colorectal cancer patients groups ($r_s = 0.494$, $P = 0.0001$ and $r_s = 0.293$, $P = 0.004$, respectively), while a positive correlation existed in both *H. pylori* seronegative and seropositive control individuals ($r_s = 0.652$, $P = 0.0001$ and $r_s = 0.381$, $P = 0.002$, respectively). Similarly, the correlation of the same parameters in *H. pylori* seropositive cancer patients was also significant ($r_s = 0.374$, $P = 0.002$, respectively).

DISCUSSION

Neopterin is mainly synthesized by activated monocytes/macrophages in response to induction by IFN- γ . Measurement of neopterin concentrations in body fluids provides information about T helper

cell 1 (Th1)-derived cellular immune activation^[20]. An increase in neopterin concentrations during cancer growth indicates a chronic cellular immune response; however, it is not specific for malignant cell proliferation^[21]. The mean serum neopterin level in the non-tumor group was below the standard cut-off value, 10 nmol/L^[22], while there was a significant increase in serum neopterin in cancer patients. Thus in our study, a highly significant increase in serum neopterin concentration of *H. pylori* seropositive colorectal cancer patients suggested that *H. pylori* seropositivity induced activation of cell-mediated immunity, in addition to cancer-induced chronic cellular immune response.

On the other hand, chronic stimulation of Th1-mediated immunity may also cause enhanced IDO activity in malignant diseases^[23]. IDO is an enzyme ubiquitously distributed in mammalian cells, and converts Trp to N-formylkynurenine. This substance is further catabolized to Kyn^[13]. Trp depletion as well as the accumulation of its metabolites results in a strong inhibitory effect on the development of immune responses^[24]. IDO-induced Trp depletion from the tumor microenvironment could be the result of enhanced activation of the enzyme and augmented Trp consumption by both tumor cells and antigen-presenting cells of the host^[25]. Recent data obtained from tumor models demonstrated that IDO inhibition could significantly enhance the antitumor activity of various chemotherapeutic and immunotherapeutic agents. These results were consistent with data showing that increased IDO expression was an independent prognostic variable for reduced overall survival in cancer patients^[24]. In colorectal cancer patients, significantly accelerated degradation of Trp, with lowered serum concentrations of Trp and increased Kyn, as well as an increased Kyn/Trp has previously been reported^[26]. Indeed, in our study, the highly significant correlation between neopterin concentrations with increased Kyn/Trp clearly indicated that the formation of Kyn is related to IDO activity by IFN- γ stimulation. It is postulated that IDO limits immune cell proliferation by depleting locally available Trp and/or producing its cytotoxic metabolites^[27]. However, enhanced IDO activity and further serum Trp degradation due to *H. pylori*-seropositivity in colorectal cancer patients was demonstrated for the first time in our study. In addition to reduced serum Trp concentrations and raised serum Kyn levels in the *H. pylori* seropositive colorectal cancer group, a significant increase in serum Kyn/Trp ($u = 1026$, $P = 0.0001$) and highly significant correlation between the serum neopterin and Kyn/Trp suggested that IDO activities may be induced by *H. pylori* seropositivity. A frequency of increased Kyn/Trp of 57% indicated that enhanced IDO activity may be an important additional factor in the development of *H. pylori*-associated colorectal cancer. As a result, *H. pylori* seropositivity may have an effect on the enhancement of immune tolerance against cancer cells. The number of advanced cancer

cases in the *H. pylori* seropositive group may support this statement.

In our study, while serum nitrite levels were significantly lower in *H. pylori* seropositive cancer patients, no difference was found between *H. pylori* seronegative cancer patients and their *H. pylori* seronegative matched controls. Thus, the nitrite-NO pathway may be viewed as a backup system, to ensure sufficient NO generation along the entire physiological oxygen gradient^[28]. Actually, NO regulates IDO activity biphasically, in a dose-dependent manner such that, while high NO production inactivates IDO enzyme and favors the immune response, low concentrations of NO increase IDO activity, resulting in immune tolerance^[29]. A decrease in serum NO in *H. pylori* seropositive cancer patients may be attributed to the relatively effective scavenging of the radicals by NO. NO and superoxide can antagonize each other's biological actions regarding the NO/superoxide balance in cytoplasmic fractions^[30]. The relative NO production rates have an impact on the NO-mediated toxic vs protective effects^[31]. Peroxynitrite formed *in vivo* from superoxide and NO can mediate oxidative nitration or nitrosation reactions, leading to tissue injury. Consequently, a reduction in NO may be due to the formation of peroxynitrite and other reactive nitrogen species (RNS). It was demonstrated that excessive reactive oxygen species (ROS)/RNS production in the *H. pylori*-infected stomach by activated neutrophils and *H. pylori* itself can damage DNA in gastric epithelial cells, implying its involvement in gastric carcinogenesis^[32]. A negative correlation between the amount of superoxide radicals and nitrites suggests that NO has antioxidative effects at the site of injury^[33]. However, the vast majority of the studies have employed serological surveillance rather than isolation of *H. pylori* from the target disease site, as serological testing for indirectly detecting *H. pylori* is quick, relatively cheap and specific. Moreover, it is well known that there is an ongoing risk of developing cancer even after the eradication of *H. pylori* infection^[34]. These data claimed that *H. pylori* may induce oxidative stress by different mechanisms. Most probably NO combines with reactive oxygen metabolites to form RNS, such as nitrogen dioxide and peroxynitrite, whereby NO bioavailability was decreased.

As we could not find any difference between the serum nitrite levels of *H. pylori* seronegative colorectal cancer cases and their matched controls; it seems that the presence of *H. pylori* may have caused a significant increase in NO consumption by formation of redox active radicals in cancer cases. H4-bip is an indispensable cofactor for NO generation by inducible nitric oxide synthase (iNOS)^[35]. The quantity of urinary biopterin excretion is a determinant of the amount of intracellular H4-bip which is critical for iNOS-dependent generation of NO^[36]. However, we did not observe an increase in urinary biopterin excretion of *H. pylori* seropositive cancer group.

In our study, a significant increase in serum neopterin concentrations suggested that IFN- γ -induced guanosine triphosphate (GTP) cyclohydrolase I activities largely supported neopterin synthesis in either *H. pylori* seronegative or *H. pylori* seropositive colorectal carcinoma patients. GTP cyclohydrolase I activities correlate with the sum of neopterin plus biopterin rather than with neopterin or biopterin alone^[37]. As a conclusion, *H. pylori* seropositive colorectal cancer patients with significantly higher Kyn/Trp and reduced NO suggest that *H. pylori* may support immune tolerance leading to cancer development, even in patients without an apparent upper gastrointestinal tract disease. There is little in the literature related to this subject, thus further studies in larger populations are warranted in order to support these findings.

COMMENTS

Background

Persistent inflammation of the stomach induced by *Helicobacter pylori* (*H. pylori*) can have consequences on the rest of the body. Despite the vigorous innate and adaptive immune response against the bacteria, it is still not known how *H. pylori* escape and evade host responses. Low tryptophan levels and increased concentrations of its degradation product, kynurenine, may be directly involved in diminished T-cell responsiveness to antigenic stimulation in cancer.

Research frontiers

An increased kynurenine to tryptophan ratio that is correlated with neopterin concentrations may be directly involved in T-cell unresponsiveness to cancer cells. The present study was designed to determine the effects of serum kynurenine/tryptophan, serum tryptophan, kynurenine and neopterin levels with persistent *H. pylori* seropositivity in colorectal cancer patients.

Innovations and breakthroughs

H. pylori seropositive colorectal cancer patients with significantly higher kynurenine/tryptophan and reduced nitric oxide suggested that *H. pylori* may support immune tolerance leading to cancer development, even in patients without an apparent upper gastrointestinal tract disease.

Applications

H. pylori seropositivity may have consequences not only in the stomach but also in the colon and rectum, and this may lead to the increased incidence of colorectal cancer. Significantly higher kynurenine/tryptophan and reduced nitric oxide may be an indicator of immune tolerance related to *H. pylori* seropositivity that supports colorectal cancer development.

Terminology

The presence of *H. pylori* immunoglobulin G is associated with previous infection. Indolamine-2,3-dioxygenase is an enzyme, ubiquitously distributed in mammalian cells, which converts tryptophan into kynurenine. The correlation of kynurenine/tryptophan with neopterin indicates indolamine-2,3-dioxygenase activity. Thus, tryptophan depletion as well as the accumulation of its metabolites results in a strong inhibitory effect on the development of immune responses against cancer cells.

Peer-review

This is a good descriptive study in which the authors analyzed consequences of *H. pylori* on the body other than stomach. The results are interesting and suggest that increased kynurenine/tryptophan may be directly involved in diminished T-cell responsiveness to antigenic stimulation in cancer and *H. pylori* might support immune tolerance leading to cancer development, even in patients without an apparent upper gastrointestinal tract disease.

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Prospective Study

Colorectal cancers in ulcerative colitis from a low-prevalence area for colon cancer

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Abstract

AIM: To determine the incidence and risk factors for colorectal cancer (CRC) in patients with ulcerative colitis from a low prevalence region for CRC.

METHODS: Our prospective database yielded a cohort of 430 patients [age: 44 ± 14.6 years; 248 men (57.7%)] with ulcerative colitis (median disease duration 6, range: 1-39 years) for analysis. Of these, 131 (30.5%) had left-sided colitis and 159 (37%) extensive colitis. Patients with histologically confirmed CRC within the segment with colitis were compared with those without CRC, to determine the risk factors for the development of CRC.

RESULTS: Twelve patients (2.8%) developed CRC. The overall incidence density was 3.56/1000 patient-years of disease - 3/1000 in the first 10 years, 3.3/1000 at 10 to 20 years, and 7/1000 at > 20 years. Three of our 12 patients developed CRC within 8 years of disease onset. On univariate analysis, extensive colitis, longer duration of disease, and poor control of disease were associated with development of CRC. On multivariate analysis, duration of disease and extent of colitis remained significant.

CONCLUSION: CRC occurred in 2.8% of patients with ulcerative colitis in our population - an incidence density similar to that in Western countries in spite of a low overall prevalence of colon cancer in our population.

The risk increased with extent and duration of disease.

Key words: Colon cancer; Dysplasia; Epidemiology; Inflammatory bowel disease; Malignancy

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Core tip: From an area with low prevalence of colon cancer, the risk of colorectal cancer (CRC) in patients with ulcerative colitis was as high as in those with high risk of CRC. Some patients developed CRC before the recommended commencement of colonoscopic surveillance for CRC.

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INTRODUCTION

The risk of development of colorectal cancer (CRC) in patients with ulcerative colitis varies in literature. A meta-analysis by Eaden *et al*^[1] in 2001 concluded that the cumulative probability of CRC was 2% by 10 years, 8% by 20 years, and 18% by 30 years. The meta-analysis by Lutgens *et al*^[2] shortlisted eight studies from 1988 to 2009 and reported that the risk of CRC is increased in inflammatory bowel disease but is not as high as reported in earlier studies; the pooled standardized incidence rate (SIR) was 1.7 (95%CI: 1.2-2.2).

These studies come from regions where the prevalence of CRC itself is high. India has an incidence of CRC that is approximately a tenth of that in the Western world^[3]. It would be interesting to see whether the intrinsically lower risk in the population would translate to a lower overall risk in patients with ulcerative colitis. Previous studies from India do indeed point to a lower risk^[4,5]; however, the duration of ulcerative colitis in the patient population evaluated has been low, with only a small percent exceeding 10 year follow up. The recent Asia-Pacific consensus statement on ulcerative colitis highlighted the paucity of data on CRC in the Asian population^[6].

In an attempt to diagnose CRC early, various societies have proposed surveillance guidelines^[6-8]. Survival benefit from colonoscopic surveillance programmes in ulcerative colitis has not been conclusively established, but there seem to be fewer deaths in patients undergoing surveillance^[7-9]. Surveillance guidelines in an otherwise low-risk population should depend on the risk increase of CRC with ulcerative colitis.

We therefore analysed the incidence of CRC in our

cohort of patients with ulcerative colitis, in order to identify risk factors and also to determine whether standard recommendations for time-to-surveillance are reasonable for low-risk populations.

MATERIALS AND METHODS

This is an analysis of a prospectively maintained database of a cohort of patients with ulcerative colitis presenting to the Division of Gastroenterology since 2005. The data include demography, history, examination findings, laboratory investigations, colonoscopy (patients with disease proximal to splenic flexure were considered to have extensive colitis) and histology findings, imaging findings, diagnosis, therapy (medical and surgical), duration of disease and therapy, compliance with therapy (taking more than 85% of the prescribed dose of medications was considered "compliant with treatment"), response to treatment, course, complications of disease, and extra-intestinal manifestations.

Currently, colonoscopic surveillance is advised routinely to our patients with more than 8 years' history of ulcerative colitis, irrespective of extent of disease. Follow up was recorded during their hospital visits, failing which they were contacted by telephone, e-mail or post, for update on their disease status. Patients with less than one-year duration of ulcerative colitis and those with less than one year of follow up were excluded from analysis in this study. Disease control was considered good when bowel frequency was normal, and there was no blood in stool and no systemic symptoms; when they were symptomatic, Truelove and Witt's criteria^[10] were used to assess disease severity; mild and moderate activity was considered as average control and severe activity was considered as poor control.

Data of patients with confirmed diagnosis of CRC on endoscopic biopsy and/or operative specimens were analysed with regard to details of location of malignancy, whether it occurred in the segment with macroscopic colitis, stage of disease, presence of metastatic disease, and outcome. These patients were compared to those without malignancy to identify possible risk factors for development of malignancy.

Statistical analysis

Qualitative data are represented as frequency and percentage. Association between qualitative variables was assessed by χ^2 test or Fisher's exact test. *P* value less than 0.05 was taken as significant. All analyses were done using SPSS Version 13.0.1, IBM, New York. The study was approved by institutional review board

RESULTS

Of the 461 patients with ulcerative colitis in our database, 31 were excluded from analysis (less

Table 1 Comparison of ulcerative colitis patients with and without colorectal cancer

	No malignancy	CRC	P value
Number of patients (males)	418 (242)	12 (5)	0.375 ¹
Age (mean \pm SD, yr)	44.5 \pm 14.6	49.6 \pm 10.1	0.23 ²
Median duration of disease (interquartile range)	6 (7)	18 (8)	0.00001 ³
History of smoking	31	0	1.0
Family history of IBD	24	2	0.15
Pancolitis	159	9	0.01
Left-sided colitis	129	2	1.0
No. of patients on azathioprine	108	2	0.738 ¹
Poor compliance with therapy	144	6	0.23
Poor disease control	50	5	0.007 ¹

¹Fisher's exact test; ²Unpaired *t* test; ³Mann Whitney test. CRC: Colorectal cancer; IBD: Inflammatory bowel disease.

than one year of duration of disease). Of the 430 patients analysed [age: 44 \pm 14.6 years; 248 males (57.7%)], 38 (8.8%) had proctitis, 95 (22.1%) procto-sigmoiditis, 131 (30.5%) left-sided colitis, and 159 (37%) extensive colitis; disease extent was not recorded in 7 patients but all these had extent beyond the rectum. The duration of ulcerative colitis was 1 to 10 years in 301 (70%) patients, 11 to 20 years in 107 (24.9%), and 21 or more years in 22 (5.1%) patients. The median duration of disease was 6 (range: 1-39 years; interquartile range 7) years.

All except three patients received 5-aminosalicylic acid (5-ASA) formulations; 109 (25.3%) patients also received azathioprine. Two hundred forty-four patients were compliant with medications. Disease control was good in 156 (36.3%), poor in 55 (12.8%), and average in the remaining. Thirty-two (7.4%) patients underwent proctocolectomy for suboptimal disease control.

Development of CRCs

Twelve patients (2.79%) developed CRC and six developed non-colorectal malignancies (one each with acute myeloid leukaemia, carcinoma breast, cholangiocarcinoma, endometrial carcinoma, laryngeal cancer and non-Hodgkin lymphoma). The risk of CRC was higher in patients with pancolitis (9/159; 5.6%) than with the other extents of disease (3/130; 2.3%) ($P = 0.0125$). One CRC was detected during surveillance at 11 years whereas others were detected during work-up for symptoms. The overall incidence density of CRC was 3.6 per 1000 person-year disease (PYD): 2.3/1000 PYD in the first 10 years, 3.3/1000 PYD in the second decade, and 7/1000 PYD thereafter. Three of 12 patients developed CRC within 8 years of disease onset (one patient had lung metastases). Associated primary sclerosing cholangitis was present in four patients - one developed cholangiocarcinoma and one CRC. No patient reported the occurrence of CRC in a first-degree relative.

Table 2 Characteristics of colorectal cancer in ulcerative colitis

No.	Age (yr)	Sex	Extent of disease	Duration ¹ , yr	Location of CRC
1	59	M	Ext colitis	24	Recto-sigmoid
2	47	F	Left-sided colitis	10	Left colon
3	51	M	Ext colitis	6	Ascending, transverse, descending colon (multifocal)
4	42	M	Ext colitis	7	Rectum
5	64	F	Ext colitis	13	Ascending colon
6	41	F	Ext colitis	17	Descending colon
7	40	F	Left-sided colitis	6	Rectum
8	46	F	Ext colitis	20	Rectum
9	67	F	Left sided	27	Rectum
10	49	M	Ext colitis	22	Rectum
11	56	M	Ext colitis	17	Rectum
12	51	F	Ext colitis	13	Ascending colon

¹Duration from onset of ulcerative colitis to detection of CRC. Ext: Extensive colitis; CRC: Colorectal cancer.

Analysis of factors affecting the development of CRC

Table 1 compares patients with and without CRC. For univariate analysis, age, gender, duration of disease, extent of colitis, history of smoking, family history of inflammatory bowel disease, medication compliance, and disease control were included. Pancolitis ($P = 0.012$), longer duration of disease ($P = 0.00001$), and poor control of disease ($P = 0.007$) were associated with development of CRC. On multivariate analysis, longer duration of disease ($P = 0.01$) and pancolitis ($P = 0.027$) were significant factors for development of malignancy.

Details of CRCs

Table 2 shows details of the patients with CRC. Malignancy developed at a median of 18 (range: 6-27 years; IQR 8) years after the onset of ulcerative colitis. Tumours were located in the rectum in six patients, recto-sigmoid junction in one, descending colon in one, ascending colon in two, and left colon in one patient. Two patients had multifocal tumours: one had 3 tumours (one each in the ascending, transverse and descending colon), and the other patient had 2 tumours (one each in the ascending colon and at the hepatic flexure).

In three patients, CRC developed with disease duration of less than 8 years. The first patient (aged 51 years) with pancolitis developed CRC after 6 years of disease and had 3 tumours. The second patient (aged 42 years), also with pancolitis, had adenoma in the rectum but refused surgery for 2 years. Two years later (7 years' disease duration) he agreed to surgery when biopsy showed adenocarcinoma in the adenoma. The third patient (aged 40 years) with left-sided colitis was incidentally detected to have lung metastases when she underwent high-resolution CT scan of the chest (at disease duration of 6 years) as part of a

study protocol; she was then found to have rectal adenocarcinoma on colonoscopy.

The pathological stage of CRC was known in 10 patients (one patient underwent surgery at another centre and one patient refused surgery and was subsequently lost to follow up): 3 patients had T1 N0 M0 stage, one patient had T2 N0 M0 disease, one had T3 N0 M0, and five had nodal involvement. One patient had lung metastases at presentation.

DISCUSSION

Our study found a prevalence rate of 2.8% for colon cancer in a cohort of 430 patients with ulcerative colitis. This is similar to that reported in previously published studies, predominantly from the Western world, where prevalence rates of CRC in ulcerative colitis varied from 0.7% to 3.3%^[11-16].

The trend in Asia is not clear; studies have shown that the likelihood of CRC is low, ranging from 0.8% to 1.8%^[4,5,17-21]. Our study, taken together with two others from India^[4,5], provides insight into the risk of CRC in ulcerative colitis in India. Kochhar *et al.*^[4] reported that the risk of CRC in ulcerative colitis was 1.8%; Venkataraman *et al.*^[5] reported a lower rate of CRC (0.94%). Our study reports a higher rate (2.79%). This is possibly a result of having a greater number of patients with duration of ulcerative colitis greater than 20 years. The duration of ulcerative colitis has not been specified in the study by Kochhar *et al.*^[4], and the lower mean duration of follow up (6 years) in the study from Vellore^[5] with no increase in incidence density between 10 and 20 years also suggests a shorter disease duration. Colectomy rates were similar in the two studies (8.8% in the Vellore study^[5] and 7.4% in ours) and could not account for the difference.

Two recent studies from the West have reached different conclusions about the increased risk of colon cancer in ulcerative colitis. Jess *et al.*^[22], in a population-based study from Denmark, suggested that the risk of colon cancer in ulcerative colitis is not as high as previously reported and in fact may not be different than that in the general population. To the contrary, Herrinton *et al.*^[23], from California showed that the risk of CRC in ulcerative colitis is 60% higher than in age- and gender-matched cohorts of people without inflammatory bowel disease, and the risk remained the same throughout the study period of 14.5 years.

Recent data suggest that the age-adjusted rates of CRC in the general Indian population vary from 2.65 to 3.06/100000 in men and 3.40/100000 in women^[3,24,25]. The low prevalence of colon cancer in the general population can be seen by the absence of a single case of colon cancer amongst first-degree relatives of our cohort of patients. The risk of CRC in Indian patients with ulcerative colitis thus appears much higher (900 times) than in the general population. This is a much larger risk factor than that in West, owing to the far lower prevalence of colon

cancer in the Indian population. This supports the contention that ulcerative colitis is a risk factor for tumour and therefore requires surveillance. If we look at the reason for relatively high prevalence of CRC in an area with low prevalence for CRC, there are no clear answer but some questions arise: It may be related to prevalence of UC in India. A study from India has shown that the incidence and prevalence of UC (incidence 6.02/100000 and prevalence 44.3/100000) is comparable to the west^[26]. The prevalence is higher than the rest of Asia^[27]. The studies on migrant Indian in Leicestershire have suggested that Indian may be more susceptible to inflammatory bowel disease (IBD) than the caucasians (RR = 2.45)^[28,29]. Secondly more than prevalence, it may be related to disease phenotypes in Indian patients. There is mixed literature on this. A study by Walker *et al.*^[30] compared the IBD disease phenotypes between South Asians (India, Pakistan and Bangladesh) and Northern Europeans living in London. The phenotype of ulcerative colitis differed significantly: higher number of South Asian patients had extensive colitis as compared to Northern European patients (63% vs 42.5%, $P = 0.0001$); The colectomy rate was non significantly lower in migrant population; they did not study the cancer development. Another study reported in an abstract form compared UC phenotype in native (living in Nagpur, Central India) Indian, Indians migrated to the United States of America (Indian-American) and Caucasian Americans^[31]. Proportion of patients with pancolitis was 34.7% in Indians, 65.9% in Indian Americans and 62.9% in Caucasian Americans. Indian Americans were more likely to have colectomy than Indian in India. So the prevalence and disease phenotypes do not seem to explain the high likelihood of CRC. Another factor like disease control may explain the high likelihood of cancer.

Our study showed that pancolitis and duration of disease are significantly associated with increased likelihood of CRC, in keeping with previous literature. Age, gender, history of smoking, family history of inflammatory bowel disease, duration of 5-ASA therapy, azathioprine therapy and compliance with therapy were not significant associations.

Guidelines by various societies suggest that surveillance for CRC should begin after 8 to 10 years of disease duration^[7-9]. However, if these recommendations are followed, CRC may be missed. Three of 12 patients with CRC in our study developed the malignancy at 6, 6 and 7 years; one of them had lung metastases at presentation. In another Indian study, by Kochhar *et al.*^[4], 2 of 8 patients with ulcerative colitis developed CRC at 7 and 8 years' disease duration. In the study by Gilat *et al.*^[17], 2 of 26 patients who developed CRC in ulcerative colitis had disease duration less than 10 years (6 and 9 years). In the study by Gong *et al.*^[21], cumulative risk of CRC in the first decade was 1.15 %, similar to the 1.6 % in the meta-analysis by Eaden *et al.*^[11], Lutgens *et al.*^[32] reported that 15% of their

patients with ulcerative colitis developed CRC before the recommended surveillance. A recent analysis from Surveillance, Epidemiology and End Result (SEER) data suggested an increased rate of missed CRC in older patients with inflammatory bowel disease^[33]. It is not clear if this can be applied to patients in other age groups with ulcerative colitis but it has relevance to surveillance strategy. Thus, although a strong body of literature suggests that few patients develop CRC with ulcerative colitis disease duration less than 10 years, it appears that approximately 10% to 20% of cancers may occur earlier during the course of the disease.

In summary, 12 (2.8%) patients with ulcerative colitis in our study developed CRC during a mean follow up of 7.8 years. The overall incidence density of cancer was 3.6 per 1000 person-year disease, with the incidence increasing with each decade. Extensive disease and duration of disease were significant risk factors for the development of CRC. Two patients had multifocal tumours; four of nine patients had nodal involvement, and one had metastases at presentation. A fourth of our patients developed cancer with disease duration less than 8 years. This study points to a significant increase in the incidence of colon cancer in ulcerative colitis over the population incidence and supports the recommendation for screening patients with ulcerative colitis even in a low-endemicity zone for colon cancer.

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COMMENTS

Background

This study presents the prevalence of colorectal cancer (CRC) ulcerative colitis from an area with low prevalence for colon cancer.

Research frontiers

The epidemiology of CRC in ulcerative colitis is changing with many studies suggesting lower CRC rates. This study suggests that CRC prevalence in ulcerative colitis patients from an area with low prevalence for CRC is equivalent to that in the areas with high prevalence for CRCs. The disease extent, severity and other factors do not explain this fully.

Applications

To study the differences in epidemiology in CRC in patients with ulcerative colitis in different parts of and to look at the innovative ways of colonoscopic surveillance in ulcerative colitis.

Peer-review

In this manuscript, the authors assessed the incidence and risk factors for CRC in patients with ulcerative colitis from a low prevalence region for CRC. They concluded that an incidence density of CRC in population with a low overall prevalence of colon cancer similar to that in Western countries, and the risk increased with extent and duration of disease. Their conclusions are reliable. Similar article however, the context lacks of novelty and can not provide new insights into CRC.

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Prospective Study

Differences in clinical features of Crohn's disease and intestinal tuberculosis

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Abstract

AIM: To investigate the clinical features of Crohn's disease (CD) and intestinal tuberculosis (ITB) with a scoring system that we have developed.

METHODS: A total of 25 CD and 40 ITB patients were prospectively enrolled from August 2011 to July 2012.

Their characteristics and clinical features were recorded. Laboratory, endoscopic, histologic and radiographic features were determined. The features with a high specificity were selected to establish a scoring system. The features supporting CD scored +1, and those supporting ITB scored -1; each patient received a final total score. A receiver operating characteristic (ROC) curve was used to determine the best cut-off value for distinguishing CD from ITB.

RESULTS: Based on a high specificity of differentiating between CD and ITB, 12 features, including longitudinal ulcers, nodular hyperplasia, cobblestone-like mucosa, intestinal diseases, intestinal fistula, the target sign, the comb sign, night sweats, the purified protein derivative test, the interferon- γ release assay (T-SPOT.TB), ring ulcers and ulcer scars, were selected for the scoring system. The results showed that the average total score of the CD group was 3.12 ± 1.740 , the average total score of the ITB group was -2.58 ± 0.984 , the best cutoff value for the ROC curve was -0.5, and the diagnostic area under the curve was 0.997, which was statistically significant ($P < 0.001$). The patients whose total scores were higher than -0.5 were diagnosed with CD; otherwise, patients were diagnosed with ITB. Overall, the diagnostic accuracy rate and misdiagnosis rate of this scoring system were 97% and 3%, respectively.

CONCLUSION: Some clinical features are valuable for CD and ITB diagnosis. The described scoring system is key to differentiating between CD and ITB.

Key words: Crohn's disease; Intestinal tuberculosis; Clinical features; Differential diagnosis; Scoring system

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Core tip: Using various traditional diagnostic methods

and currently emerging techniques, such as computed tomography enterography, this large-sample, prospective study identified specific indicators for differential diagnoses of Crohn's disease and intestinal tuberculosis and used these indicators to establish a highly valuable scoring system for differential diagnosis.

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INTRODUCTION

Crohn's disease (CD) and intestinal tuberculosis (ITB) are frequently misdiagnosed due to their high similarity in clinical manifestations^[1-7]. In spite of new emerging diagnostic techniques and recent advances in the exploration of the pathogenesis of both diseases, the specific diagnostic indicators for a differential diagnosis of those two diseases remain to be further developed.

Both CD and ITB share some symptoms, including abdominal pain, diarrhea, fever and weight loss, but they lack specificity. Lee *et al*^[8] argued that the T-SPOT.TB test (T-SPOT.TB is an interferon-gamma release assay that detects T-cell responses to early secreting antigen target 6 and culture filtrate protein 10 peptides by enzyme-linked immunospot assay for tuberculosis diagnosis. The test is highly sensitive and specific and is not affected by the subject's immune status or bacillus Calmette-Guerin vaccination.) was highly valuable for the differential diagnosis of both diseases, given that its positive and negative predictive values were 60% and 100%, respectively, for diagnosing ITB. However, large-sample studies are still needed to confirm the diagnostic value of this technique because it has just arrived in China and has failed to be widely promoted. Some scholars believe that longitudinal ulcers, aphthous ulcers and a cobblestone-like appearance are frequently found in typical CD, and circular or linear ulcers, an open ileocecal valve opening and fixed inflammatory polyps are frequently found with an endoscopy in typical ITB^[9-11]. However, these specific indicators show low positive rates, and they are atypical findings with endoscopy when used clinically. Both CD and ITB lack specific pathological indicators. Caseous granulomas can be used as the gold standard for the diagnosis of ITB^[12], but their low positive rates make early differential diagnoses of both diseases difficult. In recent years, computed tomography enterography (CTE) has been a frequently used technique for the clinical diagnosis of CD. CTE can show abnormalities, the intestinal wall, the intestines and the intestinal mucosa, and contrast-enhanced CTE can also show the involved intestinal segments and

the range^[13]. However, this technique is just emerging in China, and its diagnostic value requires the same large-sample research and confirmation as T-SPOT.TB.

By using various traditional diagnostic methods and currently emerging techniques, such as T-SPOT.TB and CTE, this prospective study (including sufficient follow-up time) identified specific indicators for the differential diagnosis of CD and ITB and used these indicators to establish a highly valuable scoring system for differential diagnosis.

MATERIALS AND METHODS

Patients with CD or ITB at the gastroenterology outpatient clinic of First Affiliated Hospital of Nanchang University were enrolled from August 2011 to July 2012. All of the patients were newly diagnosed cases in our hospital and were 18-75 years old; CD patients had not received infliximab therapy or AZA/6-MP/MTX, and ITB patients had not received anti-TB treatment. The study was approved by the ethics committee of First Affiliated Hospital of Nanchang University, and informed consent was obtained from all patients.

Patients with CD or ITB who were admitted to the hospital were found in the endoscopy room or as outpatients and were divided into the CD and ITB groups. Their characteristics and clinical features were recorded. Laboratory, endoscopic, histologic and radiographic features were determined. The diagnostic indicators were identified through statistical analyses. The diagnostic indicators with a high specificity were selected to establish a scoring system. Each indicator supporting CD scored +1, and each indicator supporting ITB scored -1. Based on this system, the total score for each patient was calculated, and the best cutoff value for the diagnosis of these two diseases was calculated based on a receiver operating characteristic (ROC) curve. Patients whose total scores were higher than this value were diagnosed with CD; patients with scores lower than this value were diagnosed with ITB. Finally, the accuracy rate and misdiagnosis rate of this scoring system for the diagnosis of these two diseases were calculated.

Active or past TB lesions on chest X-rays and the purified protein derivative (PPD) test could be very helpful in making a differential diagnosis between CD and ITB. In our study, all patients had chest X-rays and PPD tests, but only three patients had positive chest X-rays, which was not statistically significant ($P > 0.05$). Therefore, we did not select this test to establish our scoring system. There were 41 patients with positive PPD tests, which was statistically significant ($P < 0.05$), so we selected this test.

For PPD testing, 1 mL tuberculin purified protein derivative and 1 mL physiological saline solution were used. Using a 1-mL syringe, 0.1 mL concentrate was removed and diluted with saline to 0.25 mL, 0.1 mL of which was then intradermally into the medial forearm of the patient. The result is positive (+) if the

Table 1 General information about Crohn's disease and intestinal tuberculosis *n* (%)

	Gender		Age (yr)	Duration (mo)	Profession		
	Male	Female			Students and workers	Farmers	Others
CD, <i>n</i> = 25	18 (72)	7 (28)	28.57 ± 12.713	29.36 ± 42.423	15 (60)	5 (20)	5 (20)
ITB, <i>n</i> = 40	21 (53)	19 (47)	39.69 ± 13.172	17.07 ± 25.624	10 (25)	15 (38)	15 (38)
<i>P</i> value	0.118		0.248	0.378	0.005	0.137	0.137

Data are expressed as *n* (%) or the mean ± SD. CD: Crohn's disease; ITB: Intestinal tuberculosis.

scleroma diameter is more than 5 mm but less than or equal to 10 mm and positive (++) if the scleroma diameter is more than 10 mm but less than or equal to 20 mm. The test is strongly positive (+++) if the scleroma diameter is more than 20 mm or has local blisters, necrosis or lymphangitis and is negative if the scleroma diameter is less than 5 mm.

Diagnostic criteria for ITB and CD

A diagnosis of CD was made according to the World Health Organization diagnostic criteria based on clinical, radiographic, colonoscopic, and histologic features and the criteria previously established in the literature^[14]. A diagnosis of ITB was made according to the following criteria: (1) the identification of *Mycobacterium tuberculosis* by acid-fast staining or culture of biopsied specimens; (2) the presence of caseating granulomas on histological examination; and (3) an improvement of clinical and endoscopic disease activity after at least 3 mo of anti-TB therapy.

In patients in whom the differentiation between ITB and CD was uncertain, antituberculous therapy was administered for 3 mo, and the final diagnosis was made based on the clinical and endoscopic responses to antituberculous therapy. The clinical response was determined by the loss of subjective symptoms. The endoscopic response was determined by the disappearance of ulcerations.

Statistical analysis

All statistical calculations were performed using SPSS software (SPSS version 17.0; SPSS Inc., Chicago, IL, United States). Measurement data are expressed as the mean ± SD, and variables between the two groups were assessed using the *t*-test. Count data were assessed using the χ^2 test, and rates were expressed as a percentage. The total score data of the scoring system were not normally distributed, and the Wilcoxon test was used. Odds ratio (OR) values were calculated to analyze the relevance of the diagnostic criteria, and the best cutoff value for the diagnosis of these two diseases was calculated based on the ROC curve. *P* < 0.05 was considered significant.

RESULTS

General CD and ITB information

From August 2011 to July 2012, 80 patients with

suspected ITB or CD were prospectively enrolled in this study. Of the 80 patients, 12 were lost to follow-up before the diagnosis was confirmed, and 3 were diagnosed with neither CD nor ITB. Therefore, 40 patients with ITB and 25 patients with CD were analyzed in this study. The differences in gender, age and duration of disease between these two diseases were not statistically significant (*P* > 0.05). The numbers of students and workers were significantly different between these two disease groups (*P* < 0.05), but the other jobs were not significantly different between the two groups (*P* > 0.05) (Table 1).

Clinical manifestations of CD and ITB

Both CD and ITB patients had abdominal pain and weight loss, but night sweats were highly specific for ITB. None of the differences in any of the clinical manifestations were statistically significant between these two diseases (*P* > 0.05), with the exception of night sweats.

PPD, T-SPOT.TB and biochemical tests of CD and ITB

In this study, the PPD and T-SPOT.TB positive rates were higher in ITB patients than in CD patients. There were 37 PPD-positive cases (93%) and 38 T-SPOT.TB-positive cases (95%) in ITB patients and 4 PPD-positive cases (16%) and 0 T-SPOT.TB-positive cases (0%) in CD patients. Elevated high-sensitivity C-reactive protein (hs-CRP), elevated erythrocyte sedimentation rate (ESR) and decreased serum albumin were more commonly observed in CD patients, with 19 (76%), 21 (84%) and 21 (84%) cases, respectively, whereas there were 18 (45%), 18 (45%) and 21 (53%) cases, respectively, in the ITB group. The differences in all of these indicators were statistically significant between the two diseases (*P* < 0.05).

Endoscopic findings in CD and ITB

Both CD and ITB patients had multiple irregular ulcers, mainly in the terminal ileum and ileocecal valve. In CD patients, there were 13 (52%) cases of visible longitudinal ulcers (Figure 1A), 4 (16%) cases of aphthous ulcers, 13 (52%) cases of nodular hyperplasia and 6 (24%) cases of cobblestone-like mucosa. In ITB patients, there were 16 (40%) cases of visible ring ulcers (Figure 1B) and 14 (35%) cases of ulcer scars. The difference in aphthous ulcers between these two diseases was not statistically significant (*P* >

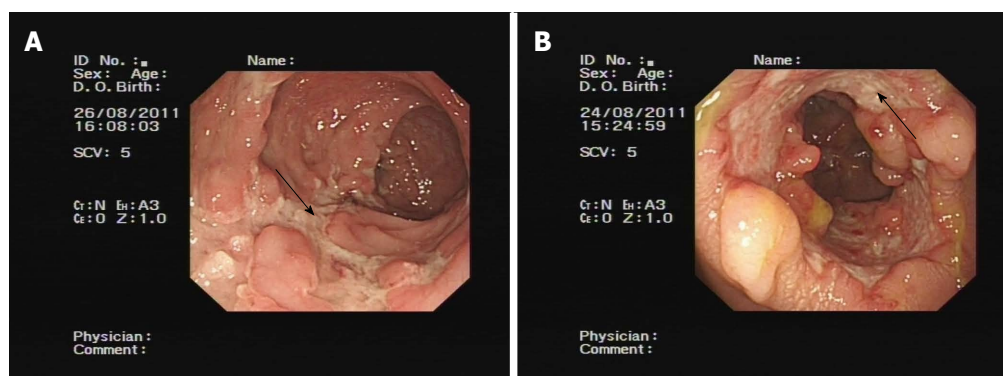


Figure 1 Longitudinal ulcer (A, arrow) and ring ulcer (B, arrow).



Figure 2 Comb sign (arrow).



Figure 3 Target sign (arrows).

0.05), and the differences in the remaining indicators were statistically significant between these two diseases ($P < 0.05$).

Pathological examination of CD and ITB

No caseous granulomas were detected in ITB patients, which is the gold standard for ITB diagnosis. There were patients with granulomas in both groups, including 15 (60%) cases from the CD group and 24 (60%) cases from the ITB group, but none of the granulomas were caseous. None of the differences in pathological indicators were statistically significant between these two diseases ($P > 0.05$).

CTE in CD and ITB

The CTE could be subjective, so two independent, blinded researchers were chosen to review each CTE. They analyzed the positive results and discussed them with the chief physician if an agreement could not be reached. The CTE results showed that the patients with CD or ITB had thickened bowel walls, so evaluations were performed to examine intestinal fistulas, target signs, comb signs and lymph node enhancement. Intestinal diseases, intestinal fistulas, comb signs (Figure 2) or target signs (Figure 3) were more commonly observed in CD patients, with 19 (76%), 9 (36%), 13 (52%) and 12 (48%) cases, respectively.

These findings were significantly different ($P < 0.05$). There were 8 cases with lymph node enhancement in the ITB group, but this result was not statistically significant ($P > 0.05$).

Value of using indicators for the differential diagnosis of CD and ITB

In our study, the following indicators were valuable for the differential diagnosis of CD or ITB: whether patients were students or workers, hs-CRP, ESR, serum albumin, longitudinal ulcers, nodular hyperplasia, cobblestone-like mucosa, intestinal diseases, intestinal fistulas, target signs, comb signs, night sweats, positive PPD tests, positive T-SPOT.TB tests, ring ulcers and ulcer scars. The OR values of whether patients were students or workers, hs-CRP, ESR, serum albumin, longitudinal ulcers, nodular hyperplasia, cobblestone-like mucosa, intestinal diseases, intestinal fistulas, target signs and comb signs were greater than 1; these indicators were risk factors of CD and were positively correlated with CD. The OR values of night sweats, positive PPD tests, positive T-SPOT.TB tests, ring ulcers, and ulcer scars were less than 1; these indicators were protective factors for CD and were negatively correlated to CD, whereas these indicators were positively correlated to ITB. The sensitivity, specificity, positive predictive value and negative predictive value of these indicators are

Table 2 Value of indicators with Crohn's disease and intestinal tuberculosis

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	OR (95%CI)
Students and workers	60%	75%	60%	75%	4.500 (1.538-13.165)
Night sweats	35%	92%	88%	47%	0.161 (0.033-0.787)
Purified protein derivative	93%	84%	90%	88%	0.015 (0.003-0.076)
T-SPOT.TB	95%	100%	100%	93%	0.050 (0.013-0.193)
High-sensitivity C-reactive protein	76%	55%	51%	79%	3.870 (1.276-11.735)
Erythrocyte sedimentation rate	84%	55%	54%	85%	6.417 (1.862-22.117)
Serum albumin	84%	48%	50%	83%	4.750 (1.380-16.353)
Longitudinal ulcer	52%	95%	87%	76%	20.583 (4.057-104.428)
Ring ulcer	40%	96%	94%	50%	0.063 (0.008-0.509)
Ulcer scar	35%	100%	100%	49%	0.650 (0.518-0.816)
Nodular hyperplasia	52%	85%	68%	74%	6.139 (1.905-19.729)
Cobblestone appearance	24%	100%	100%	68%	1.316 (1.056-1.640)
Intestinal diseases	76%	85%	76%	85%	17.944 (5.074-63.464)
Intestinal fistula	36%	95%	82%	70%	10.688 (2.074-55.081)
Target sign	52%	98%	93%	76%	42.250 (4.999-357.089)
Comb sign	48%	98%	92%	75%	36.000 (4.259-304.265)

shown in Table 2.

Scoring system for CD and ITB

Of these 16 indicators, 12 with a high specificity were selected to establish the scoring system: longitudinal ulcers, nodular hyperplasia, cobblestone-like mucosa, intestinal diseases, intestinal fistulas, target signs, comb signs, night sweats, positive PPD tests, positive T-SPOT.TB tests, ring ulcers and ulcer scars. The results showed that the average total score of the CD group was 3.12 ± 1.740 , the average total score of the ITB group was -2.58 ± 0.984 , the best cutoff value for the ROC curve was -0.5 , and the diagnostic area under the curve was 0.997 , which was statistically significant ($P < 0.001$). The diagnostic sensitivity and specificity of this scoring system were 100% and 95%, respectively. Patients whose total score was higher than -0.5 were diagnosed with CD; otherwise, they were diagnosed with ITB. The diagnostic accuracy rate and misdiagnosis rate of this scoring system were 97% and 3%, respectively.

DISCUSSION

In this study, CD was prevalent in students and workers, while ITB was prevalent in peasants, which shows that CD may be more prevalent in those who live in more developed regions, while ITB may be more prevalent in those who live in less developed regions. Due to the small sample size in this study, correlations between both diseases and living standards still require further research and confirmation. Gu *et al.*^[15] believed that bloody stools were more prevalent in patients with CD and that night sweats were more prevalent in patients with ITB. Although the presence of bloody stools was not confirmed in this study, the presence of night sweats was statistically significant. Night sweats are a typical clinical manifestation of tuberculosis and thus a useful diagnostic indicator in the differential

diagnoses of both diseases.

Li *et al.*^[16] found that the sensitivity, specificity, positive predictive value and negative predictive value of T-SPOT.TB tests were 84.2%, 75.4%, 50.0% and 94.2%, respectively. Lei *et al.*^[17] recently found specificity and positive predictive values that were also very high, which is consistent with the results of the present study. Thus, the T-SPOT.TB test plays a significant role in the differential diagnosis of both diseases. The PPD test was slightly more sensitive than the T-SPOT.TB test in this study, but the specificity of the PPD test was inferior to that of the T-SPOT.TB test, and the results of the PPD test could have been influenced by factors such as the immunity of the organism and previous BCG vaccination. Therefore, the effect of the PPD test was inferior to that of the T-SPOT.TB test. However, the PPD test, which has high sensitivity and specificity, is a helpful tool for the differential diagnosis of both diseases. In this study, the positive rates of increase in patients' hs-CRP and ESR were higher in CD, and there was greater inflammatory activity in CD. Albumin decreased in both CD and ITB, but the number of cases in which it decreased was greater in CD than in ITB, indicating that patients with CD are likely to have severe dystrophism, which contrasts with previous reports. The differences in ESR and serum albumin results may be related to severe patient conditions, a longer disease course and nutrient consumption in CD as well as to small intestine disease and relatively poor nutrition absorption. Thus, the differential diagnosis values need to be confirmed in further large-sample studies.

Endoscopy is significant for the differential diagnosis of CD or ITB and can be used for response evaluation and follow-up of both diseases. CTE can be used to identify thickened intestinal walls during the active phase of CD and intestinal diseases^[18-20], and it is helpful for the differential diagnosis of CD and ITB as well. These diseases have highly specific indicators,

such as longitudinal ulcers and ring ulcers on endoscopy and target signs and comb signs on CTE. However, their sensitivities and negative predictive values were not high, which may be related to the low incidence of CD in China and insufficient physician knowledge of endoscopic and CTE manifestations of both diseases. We must increase the sample size in future studies, reinforce learning and improve the diagnosis rate of both diseases using endoscopy and CTE.

The method used to establish the scoring system of specific diagnostic indicators in this study was introduced by Lee *et al.*^[21]. Their scoring system was based mainly on 8 specific endoscopic indicators: less than 4 lesions, a deformed ileocecal valve, ring ulcers, ulcer scars, anorectal lesions, longitudinal ulcers, aphthous ulcers and cobblestone appearance. In this study, to build a new scoring system, we added new indicators to the foundational endoscopy indicators, *i.e.*, the PPD test, the T-SPOT.TB test and CTE. Compared with previous reports, there were some differences in the indicators found in this study^[2,4,21-24], which might be related to the sample size and to regional disparity. If the studied patients had been diagnosed using the scoring system described by Lee *et al.*^[21], 56 of 65 patients would have been diagnosed correctly, 9 would have been misdiagnosed, and the accuracy and the misdiagnosis rate would have been 86% and 14%, respectively. However, the diagnostic accuracy of the scoring system established in this study was 97% for both diseases. Thus, the scoring system established in this study is more valuable than that proposed by Lee *et al.*^[21] for the differential diagnosis of CD and ITB.

The scoring system described herein, which was established based on high-specificity features, is valuable for the differential diagnosis of CD and ITB. In view of the small sample size and the equal weights assigned to all diagnostic indicators in this study, gradual improvement could be attained for this scoring system by further increasing the sample size and determining the weights for all diagnostic indicators.

COMMENTS

Background

Crohn's disease (CD) and intestinal tuberculosis (ITB) are frequently misdiagnosed due to the high similarity of their clinical manifestations. Despite new emerging diagnostic techniques and recent advances in the exploration of the pathogenesis of both diseases, the specific diagnostic indicators for the differential diagnosis of these two diseases remain to be elucidated.

Research frontiers

With the emergence of new diagnostic technology, such as the interferon-γ release assay (T-SPOT.TB test), computed tomography enterography, and magnetic resonance enterography, the diagnostic accuracy of differences between CD and ITB has increased. However, these new methods have either low sensitivity and high specificity or high sensitivity and low specificity. An investigation of the clinical features of CD and ITB using a scoring system is needed.

Innovations and breakthroughs

The aim of this study was to test the differences between CD and ITB using clinical, laboratory, endoscopic, histological and radiographic features. It is

suggested that the establishment of a scoring system based on high-specificity features would be valuable for the differential diagnosis of CD and ITB.

Applications

Some clinical features are valuable for the diagnosis of CD and ITB. Establishing a scoring system based on high-specificity features is key to differentiating between CD and ITB.

Peer-review

This prospective study was performed to develop a scoring system to differentiate between CD and ITB. The clinical significance of this type of study seems very high in Asian countries, in which ITB is still prevalent and the incidence of CD is continuously increasing. The authors have engaged this subject, which has been eluding clinicians for a long time, and have compared the results of several important parameters. The authors have found significant results that may be clinically valuable.

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Prospective Study

Early kidney injury during long-term adefovir dipivoxil therapy for chronic hepatitis B

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binding protein (RBP) excretion, and renal impairment with adefovir dipivoxil (ADV) for chronic hepatitis B.

METHODS: We enrolled 165 patients with chronic hepatitis B infection who were treated with ADV monotherapy ($n = 90$) or ADV plus lamivudine combination therapy ($n = 75$). An additional 165 chronic hepatitis B patients treated with entecavir were recruited as controls. We detected serum creatinine, urine β 2-M, and RBP levels, and estimated the glomerular filtration rate (eGFR) at the initiation of antiviral therapy and every 6 mo for a period of five years.

RESULTS: Urine β 2-M abnormalities were observed in patients during the first ($n = 3$), second ($n = 7$), third ($n = 11$), fourth ($n = 16$), and fifth ($n = 21$) year of ADV treatment. Urinary RBP abnormalities were observed in patients during the first ($n = 2$), second ($n = 8$), third ($n = 12$), fourth ($n = 15$), and fifth ($n = 22$) year of ADV treatment. eGFR decreased 20%-30% from baseline in 20 patients, 30%-50% in 12 patients, and $> 50\%$ in 3 patients during the five years of treatment. Further analysis indicated that decreases in eGFR of $\geq 30\%$ relative to the baseline level correlated significantly with urine RBP and β 2-M abnormalities. In contrast, both serum creatinine and eGFR remained stable in patients treated with entecavir, and only one of these patients developed a urine β 2-M abnormality, and two developed urine RBP abnormalities during the five years of treatment.

CONCLUSION: Urine RBP and β 2-M are biomarkers of renal injury during long-term ADV treatment for chronic hepatitis B, and indicate when treatment should be switched to entecavir.

Key words: Adefovir dipivoxil; Entecavir; Retinol binding protein; Renal impairment; Urine β 2-microglobulin

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Abstract

AIM: To evaluate urine β 2-microglobulin (β 2-M), retinol-

Core tip: Identifying a reliable and sensitive biomarker of early renal dysfunction would be helpful for facilitating early intervention and evaluating the effectiveness of treatments for chronic hepatitis B. Urinary β_2 -microglobulin and retinol-binding protein are early markers of nephrotoxicity induced by nephrotoxic substances, cardiac surgery, diabetes mellitus, or hypertension. This study shows that long-term adefovir dipivoxil therapy in patients with chronic hepatitis B results in renal impairment that correlates with abnormalities in these markers.

Jia HY, Ding F, Chen JY, Lian JS, Zhang YM, Zeng LY, Xiang DR, Yu L, Hu JH, Yu GD, Cai H, Lu YF, Zheng L, Li LJ, Yang YD. Early kidney injury during long-term adefovir dipivoxil therapy for chronic hepatitis B. *World J Gastroenterol* 2015; 21(12): 3657-3662 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3657.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3657>

INTRODUCTION

More than 350 million people worldwide are infected with the hepatitis B virus (HBV)^[1]. Hepatitis B is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, for which high levels of HBV DNA are an independent factor^[2]. Therefore, the main goal of treatment is complete suppression of HBV replication to limit progressive liver damage and improve the natural history of chronic hepatitis B (CHB). Currently, oral nucleoside analogs have demonstrated success in suppressing virus replication, with few side effects. Evidence-based medicine has demonstrated that a slow virologic response after initiation of nucleoside analog treatment is associated with high rates of long-term drug resistance^[3,4].

Among the available nucleoside analogs, adefovir dipivoxil (ADV) is a phosphonate acyclic nucleotide analog of AMP. It is a potent inhibitor of HBV reverse transcriptase and is effective for patients with hepatitis B e antigen (HBeAg)-positive and HBeAg-negative CHB^[5,6]. ADV also shows no cross-resistance with other nucleoside analogs such as lamivudine (LAM), telbivudine, and entecavir (ETV), and thus is widely used as a rescue therapy for these drugs^[7,8]. However, renal dysfunction associated with prolonged use of ADV has been reported recently^[9-11]. For example, in a study of 10 mg ADV combined with 100 mg LAM, serum creatinine increased in 38% of patients following median treatment duration of 38 mo^[12]. However, most routine renal function tests used in these studies were based on serum creatinine, blood urea nitrogen, and estimated glomerular filtration rate (eGFR), which failed to identify early stages of renal dysfunction and structural injury. Therefore, identification of a reliable and sensitive biomarker of early renal dysfunction would be helpful for facilitating early intervention and

evaluating its effectiveness in CHB patients.

β_2 -Microglobulin (β_2 -M) is an 11.8-kDa protein, and is a light chain of major histocompatibility class I expressed on the surface of every nucleated cell^[13]. Retinol-binding protein (RBP) is a protein of 21 kDa that is synthesized by the liver. Both β_2 -M and RBP are subject to glomerular filtration, the bulk of which undergo proximal tubular reabsorption and catabolism, which might be disrupted in renal dysfunction^[14]. β_2 -M and RBP excretion in urine has been reported as an early marker of nephrotoxicity induced by nephrotoxic substances, cardiac surgery, diabetes mellitus, or hypertension^[15]. At present, it remains unclear whether urine β_2 -M and RBP can be used as early markers to diagnose renal impairment in CHB patients with long-term ADV treatment. In this study, we aimed to evaluate the relationship between urine β_2 -M and RBP excretion and early renal impairment during long-term ADV treatment in CHB patients.

MATERIALS AND METHODS

Patients

From January 2007 to March 2010, 165 patients diagnosed with CHB at the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) were treated with ADV monotherapy ($n = 90$) or ADV and LAM combination therapy ($n = 75$). An additional 165 CHB patients treated with ETV were also recruited as controls. All the patients had normal renal function at the outset of ADV and ETV treatment (serum creatinine $< 59 \mu\text{mol/L}$ and eGFR of $\geq 50 \text{ mL/min per } 1.73 \text{ m}^2$). Urinary excretion of β_2 -M and RBP was not detected in any of the patients at the beginning of the study. We excluded patients infected with hepatitis delta virus, hepatitis C virus, or those who had HIV co-infection. Patients with hypertension, diabetes mellitus, hepatocellular carcinoma, autoimmune hepatitis, alcoholic liver cirrhosis, or severe heart, renal and brain diseases were also excluded. All patients who participated in this study provided informed consent and were aware of the procedures to be conducted. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University.

Follow-up studies

Serum HBV markers, including hepatitis B surface antigen, anti-HBs, HBeAg, hepatitis B e antibody (anti-HBe), and hepatitis B core antibody, were detected by commercially available enzyme immunoassays (Abbott Laboratories, Chicago, IL, United States). Serum HBV DNA was measured by PCR with a linear range between 1×10^3 copies/mL and 5×10^8 copies/mL (Shanghai ZJ Bio-Tech Co. Ltd., China).

Patients visited our hospital every 3-6 mo after the start of ADV and ETV treatment. Follow-up clinical assessments included physical examination, HBeAg and anti-HBe, quantitative HBV DNA, serum biochemistry, α -fetoprotein, renal function, and

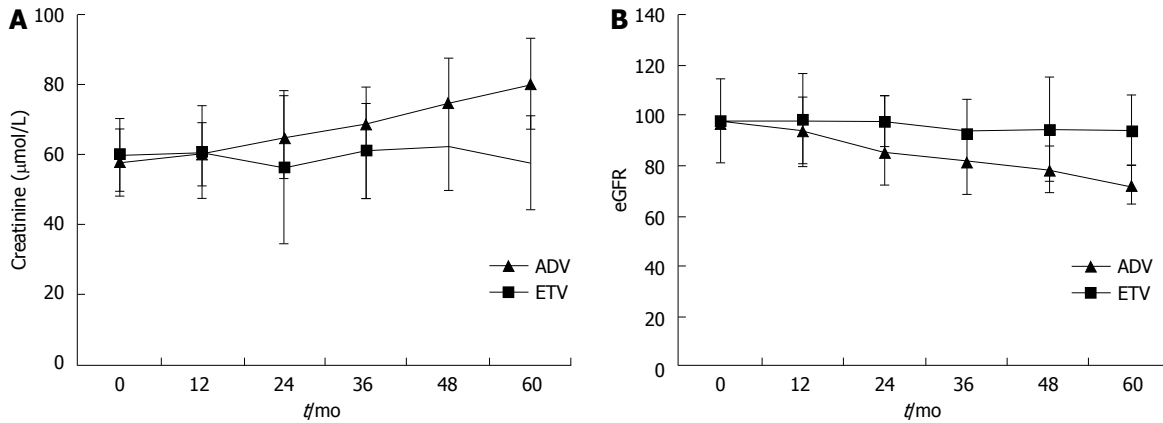


Figure 1 Time course after treatment with adefovir dipivoxil with or without lamivudine and entecavir for chronic hepatitis B patients. A: Changes in mean creatinine level; B: Mean estimated glomerular filtration rate (eGFR) with adefovir dipivoxil (ADV) and entecavir (ETV) administration.

Table 1 Baseline characteristics *n* (%)

Characteristic	ADV or ADV + LAM (<i>n</i> = 165)	ETV (<i>n</i> = 165)	<i>P</i> value
Age (yr)	46.2 ± 9.2	48.6 ± 8.7	0.89
Male	115 (69.7)	120 (72.7)	0.68
Treatment duration (mo)	54.6 (12–95)	53.8 (12–98)	0.76
HBeAg positive	95 (57.6)	93 (56.3)	0.64
HBV DNA (log10 copies/mL)	6.65 ± 0.93	6.54 ± 0.89	0.88
Total bilirubin (mmol/L)	48.9 (23–71)	45.7 (21–75)	0.76
ALT (U/L)	146 (85–210)	156 (89–216)	0.54
Albumin (g/L)	45.6 ± 6.7	44.9 ± 7.1	0.32
Creatinine (mmol/L)	57.8 ± 4.6	59.7 ± 8.3	0.45
eGFR (mL/min per 1.73 m ²)	97.8 ± 10.7	96.7 ± 13.5	0.23
Inorganic phosphate (mmol/L)	1.43 ± 0.9	1.37 ± 0.8	0.12
β2-M (g/mol Cr)	1.4 ± 0.006	1.3 ± 0.005	0.55
RBP (g/mol Cr)	1.6 ± 0.004	1.7 ± 0.008	0.51

ADV: Adefovir dipivoxil; ALT: Alanine aminotransferase; β2-M: β2-microglobulin; eGFR: Estimated glomerular filtration rate; ETV: Entecavir; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; LAM: Lamivudine; RBP: retinol-binding protein.

ultrasonography or CT. The eGFR (measured as mL/min per 1.73 m²) was calculated by the Chinese equation [$175 \times \text{Pcr}^{-1.234} \times \text{age}^{0.179}$ (female $\times 0.79$)]. Renal impairment was indicated by a decrease in eGFR to < 50 mL/min per 1.73 m². Urine β2-M and RBP were tested in the First Affiliated Hospital of Zhejiang University. The normal values of urine β2-M and RBP were 0.000–0.025 g/mol Cr, respectively.

Statistical analysis

SPSS version 16.0 software (SPSS Inc., Chicago, IL, United States) was used for data analysis. Measurements are presented as mean ± SD and comparisons were conducted using the Student's *t* test. Proportions are presented as percentages, and rate comparisons were performed using the χ^2 test. The cumulative incidence of renal impairment and urine β2-M and RBP changes were calculated using the Kaplan-Meier method, and group data were calculated

using the log rank test. The Cox proportional hazard regression model was used to estimate univariate and multivariate risk factors for urine microprotein (β2-M and RBP) abnormalities. *P* < 0.05 was considered significant.

RESULTS

ADV-related nephrotoxicity

Baseline characteristics of the groups did not differ, and are presented in Table 1. Figure 1 shows the dynamic changes in the mean value of creatinine and eGFR from baseline during ADV (with or without LAM) and ETV treatment. The creatinine level increased gradually from the second year, and was increased 20%–30% from baseline in 32/165 (19.4%) patients, 30%–50% in 15/165 (9.1%), and > 50% in 4/165 (2.4%) patients during the five-year period. Serum creatinine was > 104 μmol/L in 5/165 (3.0%) patients treated with ADV. eGFR decreased 20%–30% from baseline in 20/165 (12.1%) patients, 30%–50% in 12/165 (7.2%), and > 50% in 3/165 (1.8%) in patients treated with ADV, and three patients displayed renal impairment (eGFR < 50 mL/min per 1.73 m²). In the ETV control treatment group, both creatinine and eGFR remained stable over the five-year period.

Frequency of urine β2-M abnormality

Cumulative incidences of urine microprotein abnormalities are shown in Figure 2. Urine β2-M abnormality developed in 3/165 (1.8%), 7/165 (4.2%), 11/165 (6.7%), 16/165 (9.7%), and 21/165 (12.7%) patients in the first, second, third, fourth, and fifth year of ADV or ADV plus LAM treatment, respectively. Urine RBP abnormality developed in 2/165 (1.2%), 8/165 (4.8%), 12/165 (1.8%), 15/165 (9.1%), and 22/165 (13.3%) patients in the first, second, third, fourth, and fifth year of treatment, respectively. Only 1/165 (0.1%) patient developed a urine β2-M abnormality, and 2/165 (1.2%) patients developed urine RBP abnormalities during the five years of ETV treatment. The incidence

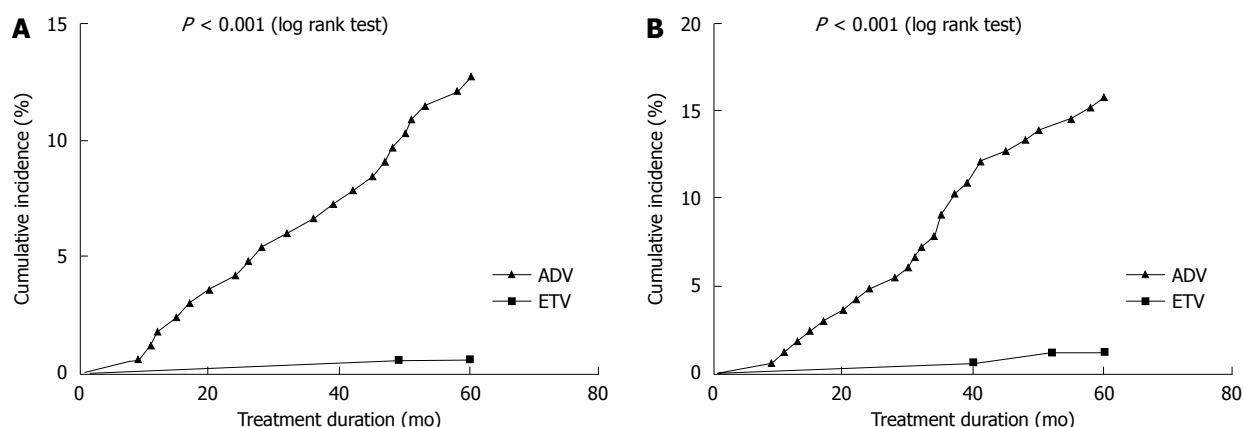


Figure 2 Cumulative incidence of urine microprotein abnormalities after treatment with adefovir dipivoxil with or without lamivudine or entecavir in chronic hepatitis B patients. Cumulative incidence of A: Urine β 2-microglobulin abnormality; and B: Retinol-binding protein abnormality with long-term adefovir dipivoxil (ADV) or entecavir (ETV) treatment.

Table 2 Determinants of urine β 2-microglobulin abnormality

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P value	HR (95%CI)	P value
Age ≥ 50 yr	5.237 (2.687-10.742)	< 0.001	3.675 (1.612-7.865)	0.003
Male	1.523 (0.876-3.487)	0.782	-	-
Body weight	1.323 (0.712-3.237)	0.574	-	-
Baseline eGFR < 80 mL/min per 1.73 m ²	3.879 (1.657-7.986)	0.001	-	-
ADV treatment	5.178 (2.358-9.867)	< 0.001	3.078 (1.328-6.871)	0.005

ADV: Adefovir dipivoxil; CI: Confidence interval; eGFR: Estimated glomerular filtration rate; HR: Hazard ratio.

of urine β 2-M abnormality in the ADV treatment group was higher than that in the ETV group ($P < 0.001$).

Further analysis indicated that a $\geq 30\%$ decrease in eGFR relative to the baseline level after five years correlated significantly with urine RBP and β 2-M abnormality ($P = 0.006$ and 0.005 , respectively).

Predictive factors for urine β 2-M abnormality

The results of univariate and multivariate analyses are indicated in Table 2. Univariate analysis showed that old age (≥ 50 years), ADV treatment, and baseline mild renal dysfunction (eGFR < 80 mL/min per 1.73 m²) were associated with the development of urine β 2-M abnormality (all $P < 0.001$). Multivariate analysis indicated that old age ($P = 0.003$) and ADV treatment ($P = 0.005$) were significant predictors of urine β 2-M abnormality.

Effect of switching ADV to ETV treatment on renal impairment

Among 22 patients with urine β 2-M abnormalities, 11 switched to ETV treatment while the other 11 continued ADV treatment. As indicated in Figure 3, both urine RBP and β 2-M decreased gradually after

switching to ETV treatment for 48 wk, while both urine RBP and β 2-M increased gradually in the ADV or ADV plus LAM treatment group. eGFR increased from 78.5 ± 11.8 mL/min per 1.73 m² to 89.5 ± 13.7 mL/min per 1.73 m² after switching to ETV treatment for 48 wk. eGFR deteriorated from 79.8 ± 14.3 mL/min per 1.73 m² to 68.5 ± 14.3 mL/min per 1.73 m² if ADV was continuously applied.

DISCUSSION

Renal dysfunction is one of the serious adverse effects of ADV. In a previous retrospective study of 687 patients, during a median treatment duration of 27 mo, 10.5% of patients developed renal impairment, which was defined as a decrease in eGFR > 20% relative to baseline^[16]. In another study of 292 patients treated with ADV plus LAM combination therapy, 9.6% patients developed renal impairment during a median treatment duration of 64.3 mo^[9]. Our results show that during the five years of ADV or ADV plus LAM treatment, creatinine increased by 20%-30% from baseline in 19.4%, 30%-50% in 9.1%, and > 50% in 2.4% of patients, while the eGFR decreased 20%-30% from baseline in 12.1%, 30%-50% in 7.2%, and > 50% in 1.8% of patients. Furthermore, five patients had serum creatinine > 104 μ mol/L and three had eGFR < 50 mL/min per 1.73 m² during the five years of ADV or ADV plus LAM treatment. These rates are less than those reported previously, because all the CHB patients with hypertension, diabetes mellitus, and liver cirrhosis were excluded from our study. In contrast to ADV treatment, both creatinine and eGFR remained stable in the ETV treatment group.

Routine renal function tests based on serum creatinine and blood urea nitrogen fail to identify early stages of renal dysfunction and structural injury. Creatinine change is not specific because it can also occur in non-renal disease, reflecting changes in muscle mass and nutrition intake^[17]. eGFR mainly reflects the renal filtration capacity, and cannot detect

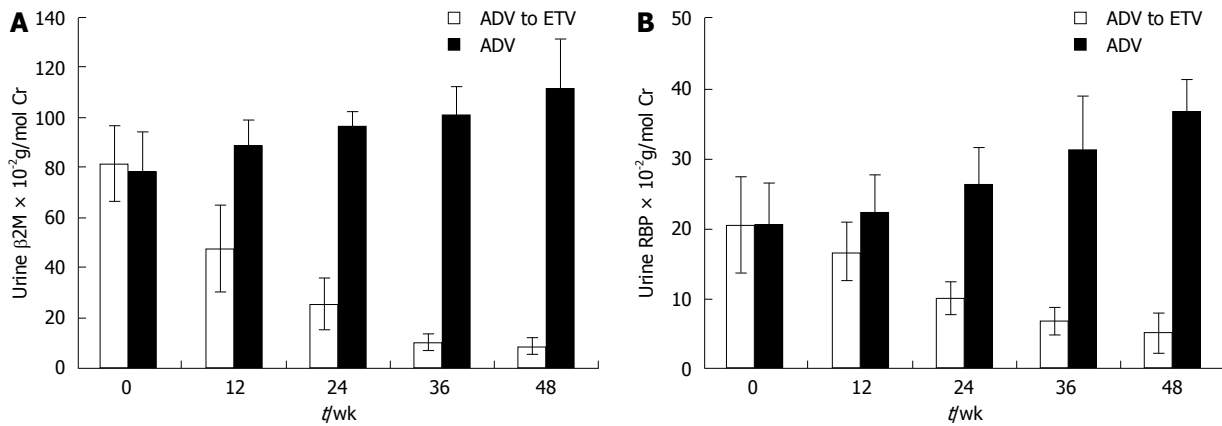


Figure 3 Changes in urine microproteins after modification of adefovir dipivoxil treatment in chronic hepatitis B patients. A: Mean urine β 2-microglobulin (β 2-M); B: Mean urine retinol-binding protein (RBP) after switching to entecavir treatment [adefovir dipivoxil (ADV) to entecavir (ETV)] or with continued ADV treatment.

the early stage of renal tubular disorders. Therefore, other biomarkers have been examined, such as β 2-M, RBP, kidney injury molecule-1, neutrophil gelatinase associated lipocalin, interleukin-18, and sodium/hydrogen exchange form 3^[18]. These biomarkers show increased levels in the urine at an early stage after renal dysfunction occurs. Our results indicate that 1.8%, 4.2%, 6.7%, 9.7% and 12.7% of patients developed urine β 2-M abnormalities, and 1.2%, 1.8%, 4.8%, 9.1%, and 13.3% of patients developed urine RBP abnormalities in the first, second, third, fourth, and fifth year of treatment, respectively. Only 3% of patients displayed serum creatinine $> 104 \mu\text{mol/L}$ and 1.8% eGFR $< 50 \text{ mL/min per } 1.73 \text{ m}^2$ at the same time. This indicates that the urine β 2-M and RBP abnormalities preceded creatinine abnormality renal impairment.

The mechanism of ADV-induced nephrotoxicity may be related to drug accumulation in renal proximal tubules after long-term administration, which may reduce the reabsorption capacity of microproteins (β 2-M and RBP), amino acids, glucose, phosphorus, and calcium^[19,20]. Detection of these biomarkers in serum or urine might be important, because at this stage, positive results should raise awareness of the risk of renal damage at the moment when it is still reversible with prophylactic or therapeutic intervention. In our study, among 11 patients with urine β 2-M abnormality, urine RBP and β 2-M decreased gradually, and eGFR increased after switching to ETV treatment for 48 wk, whereas urine RBP and β 2-M continued to increase, and the eGFR decreased in those remaining on ADV treatment. Our results strongly suggest that ADV should be switched to ETV treatment immediately if urine RBP or β 2-M is detected in CHB patients.

We also analyzed the risk factors of urine β 2-M abnormality after long-term ADV treatment. Consistent with previous reports, our univariate analysis indicated that age was a significant and independent factor of urine β 2-M abnormality, together with long-term ADV administration and baseline mild dysfunction (eGFR

$< 80 \text{ mL/min per } 1.73 \text{ m}^2$)^[9,10]. Multivariate analysis suggested that age and ADV administration were independent factors of urine β 2-M abnormality.

In conclusion, both urine RBP and β 2-M are sensitive biomarkers for detecting early renal injury during long-term ADV treatment. Therefore, when RBP or β 2-M are detected in urine, ADV should be switched to ETV immediately. Moreover, ADV should be avoided as a first-line treatment for CHB patients, especially for elderly people.

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Ding F is a graduate student, now working in the Sixth People's Hospital of Shaoxing, Zhejiang, China.

COMMENTS

Background

Renal dysfunction associated with prolonged use of adefovir dipivoxil (ADV) has been reported recently. However, most routine renal function tests used in these studies were based on serum creatinine, blood urea nitrogen, and estimated glomerular filtration rate (eGFR), which fail to identify early stages of renal dysfunction and structural injury. Therefore, identification of a reliable and sensitive biomarker of early renal dysfunction would be helpful in facilitating early intervention and evaluating its effectiveness for chronic hepatitis B (CHB) patients. The aim of this study was to evaluate the relationship between urine β 2-microglobulin (β 2-M) and retinol-binding protein (RBP) excretion and early renal impairment during long-term ADV in CHB patients.

Research frontiers

β 2-M is an 11.8-kDa protein, and is a light chain of major histocompatibility class I expressed on the surface of every nucleated cell. RBP is a protein of 21 kDa that is synthesized by the liver. Both β 2-M and RBP are subject to glomerular filtration, and undergo proximal tubular reabsorption and catabolism, which might be disrupted in renal dysfunction. β 2-M and RBP excretion in urine has been reported as an early marker of nephrotoxicity induced by nephrotoxic substances, cardiac surgery, diabetes mellitus, or hypertension. At present, it remains unclear whether urine β 2-M and RBP can be used as early markers to diagnose renal impairment in CHB patients with long-term ADV treatment.

Innovations and breakthroughs

Routine renal function tests based on serum creatinine and blood urea nitrogen fail to identify early stages of renal dysfunction and structural injury. Recently, several biomarkers have been found, such as β 2-M, RBP, kidney injury molecule-1, neutrophil gelatinase associated lipocalin, interleukin-18, and sodium/hydrogen exchange form 3, which show increased levels in the

urine at an early stage after renal dysfunction occurs. These results indicated that a proportion of patients developed urine β 2-M and RBP abnormalities during long-term ADV treatment, which occur before creatinine abnormality and impaired eGFR. Thus, detection of urine β 2-M and RBP can help identify early renal dysfunction during long-term ADV treatment.

Applications

The study results suggest that urine β 2-M and RBP can be used as early biomarkers to indicate renal dysfunction during long-term ADV treatment.

Terminology

ADV is a phosphonate acyclic nucleotide analog of AMP. It is a potent inhibitor of hepatitis B virus reverse transcriptase and is effective for patients with chronic hepatitis B infections.

Peer-review

This is a good descriptive study in which the authors analyzed the β 2-M and RBP excretion induced by long-term ADV treatment in the CHB patients. The results are interesting and suggest that urine RBP and β 2-M are sensitive biomarkers of early renal injury during long-term ADV treatment. ADV should be switched to ETV as soon as urine RBP or β 2-M is detected. ADV should be avoided as first-line treatment for CHB.

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Randomized Controlled Trial

Doctor communication quality and Friends' attitudes influence complementary medicine use in inflammatory bowel disease

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Author contributions: Mountifield R was involved in conception, design, seeking ethical approval, data acquisition and analysis, data interpretation, manuscript drafting and modification and preparation of the final paper; Andrews JM and Bampton P were involved in planning the study; Bampton P in maintaining the FMC database; and both Andrews JM and Bampton P in data interpretation and revising the manuscript; and Mikocka-Walus A assisted with data entry and analysis.

Ethics approval: This study was approved by Flinders Clinical Research Ethics Committee on behalf of SA subjects and Menzies School of Health Human Research Ethics Committee for Darwin subjects.

Informed consent: All study participants provided informed consent prior to study enrolment.

Conflict-of-interest: Réme Mountifield has received speaker fees from Ferring, AstraZeneca, and Janssen; Jane M Andrews has been an advisory board consultant for Abbvie, Schering-Plough, Ferring, Fresenius-Kabi, Janssen, Takeda, Hospira and has consulted for Orphan and Shire; Jane M Andrews also received research funding from Abbvie and Janssen and received speaker fees from Abbvie, Astra Zeneca, MSD, Fresenius Kabi, Janssen, Orohan, Nycomed, Ferring, Takeda and Shire; Antonina Mikocka Walus has received speaker fees from MSD pharmaceuticals; Peter Bampton has no conflict of interest to disclose.

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Abstract

AIM: To examine the frequency of regular complementary and alternative therapy (CAM) use in three Australian cohorts of contrasting care setting and geography, and identify independent attitudinal and psychological predictors of CAM use across all cohorts.

METHODS: A cross sectional questionnaire was administered to inflammatory bowel disease (IBD) patients in 3 separate cohorts which differed by geographical region and care setting. Demographics and frequency of regular CAM use were assessed, along with attitudes towards IBD medication and psychological parameters such as anxiety, depression, personality traits and quality of life (QOL), and compared across cohorts. Independent attitudinal and psychological predictors of CAM use were determined using binary logistic regression analysis.

RESULTS: In 473 respondents (mean age 50.3 years, 60.2% female) regular CAM use was reported by

45.4%, and did not vary between cohorts. Only 54.1% of users disclosed CAM use to their doctor. Independent predictors of CAM use which confirm those reported previously were: covert conventional medication dose reduction ($P < 0.001$), seeking psychological treatment ($P < 0.001$), adverse effects of conventional medication ($P = 0.043$), and higher QOL ($P < 0.001$). Newly identified predictors were CAM use by family or friends ($P < 0.001$), dissatisfaction with patient-doctor communication ($P < 0.001$), and lower depression scores ($P < 0.001$).

CONCLUSION: In addition to previously identified predictors of CAM use, these data show that physician attention to communication and the patient-doctor relationship is important as these factors influence CAM use. Patient reluctance to discuss CAM with physicians may promote greater reliance on social contacts to influence CAM decisions.

Key words: Complementary medicine; Alternative therapy; Therapy; Inflammatory bowel disease; Patient-Doctor Communication; Medication adherence

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Core tip: Complementary medicine use is widespread in inflammatory bowel disease, and potentially deleterious to treatment outcomes. Whilst demographic and clinical predictors of complementary and alternative therapy (CAM) are well established, attitudinal influences are under explored. This study demonstrates that the specific aspect of patient doctor relationship most influencing CAM use is quality of doctor communication. The other newly identified predictor of CAM use is its use by family and friends. This finding enables valuable insight suggesting that in the absence of good doctor communication, inflammatory bowel disease patients seek advice from unqualified sources such as family and friends.

Mountifield R, Andrews JM, Mikocka-Walus A, Bampton P. Doctor communication quality and Friends' attitudes influence complementary medicine use in inflammatory bowel disease. *World J Gastroenterol* 2015; 21(12): 3663-3670 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3663.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3663>

INTRODUCTION

The use of complementary and alternative medicine (CAM) is widespread in inflammatory bowel disease (IBD), rates ranging from 31% to 74% in studies across Europe^[1-3], Australasia^[4,5], and North America^[6]. Studies examining the efficacy and safety of these treatments in IBD are heterogenous and controlled data limited^[7], thus it is difficult for physicians to

advise patients regarding these potentially deleterious agents. However, the ongoing consumer demand for alternatives to conventional therapy means that IBD physicians need to be alert to CAM use, its associated behaviours and underlying health beliefs that may influence conventional IBD care.

Approximately three quarters of CAM taking IBD patients do not discuss its use with their IBD physician^[3,8], thus there is a need to identify surrogate markers or predictors of use that may prompt discussion about CAM during routine consultation.

Predictors previously established fall into demographic, clinical and attitudinal categories. Independent demographic predictors of CAM use include younger age^[1,5,9], female gender^[1,5,9], higher educational level^[5,9], income and employment^[5,8], and middle social class at birth^[5]. Clinical predictors are more controversial^[10,11] but have included Crohn's disease^[9], longer disease duration^[12], medication type^[1,13], active disease^[14], the experience of adverse effects of conventional medication^[2,14,15], and a concurrent diagnosis of irritable bowel syndrome (IBS)^[16].

Some studies however, have suggested that health attitudes and behaviours are more important than demographics in influencing CAM use^[15,17], and there has been recent enthusiasm to identify attitudinal and behavioural predictors as these factors are potentially modifiable. Data regarding such predictors are more limited and heterogenous but suggest that a need for control over disease^[17], desire for a holistic approach^[17], lack of confidence in the IBD physician^[17], poorer therapeutic relationships^[18], and vegetarianism^[5] are associated with CAM use. CAM use has also been suggested as a marker of psychological or social distress^[16].

Disparity in findings between different studies may relate in part to cultural differences in IBD populations, as suggested by an Italian study which demonstrated regional variations in CAM type chosen, despite similar rates of use across the cohorts^[14]. An Australian diabetes study suggested an effect of health care setting on CAM use frequency, reporting private health insurance as an independent predictor of CAM use^[19]. In IBD patients in Australia, whilst overall frequency and potential ethnically based differences in CAM use have been previously examined^[4], attitudinal and psychological predictors of its use are unexplored, as is the effect of the health care setting on CAM uptake.

MATERIALS AND METHODS

Subject selection and recruitment

IBD patients from three different care settings in two distinct geographical locations in Australia were invited to participate. This method has been reported previously^[20].

The first cohort came from a metropolitan public teaching hospital based specialist IBD Service

at Flinders Medical Centre (FMC). This is a large, government funded hospital, offering secondary/tertiary care for a local regional population of 341000 with a Gastroenterology inpatient and outpatient service, and IBD nurses available to patients within working hours.

The second cohort consisted of IBD patients in an overlapping area, receiving their care *via* a metropolitan Private Practice setting. These patients were under the care of one of four male general Gastroenterologists with extensive experience in managing IBD, without attachment to a specialist IBD unit, or access to IBD specialist nurse support.

The third cohort consisted of IBD patients cared for *via* Royal Darwin Hospital (RDH), a public hospital in a very remote location in Northern Australia. When this study was conducted, IBD care in Darwin was undertaken predominantly by general practitioners (GPs) and general surgeons, with no specialist gastroenterologist residing in Darwin, and no IBD nurse. The nearest tertiary hospital is in Adelaide, SA, more than 3000 kilometres away.

Potential subjects were identified from IBD databases/hospital records in each location and mailed a questionnaire. Reminder letters were sent to non-responders after one and three months.

Questionnaire content

The opening section of the questionnaire sought demographic details including age, gender, disease type, indigenous, relationship and employment status as well as current or previous history of smoking.

In the following sections, A-D, participants answered questions assessing: (1) views regarding conventional IBD medications; (2) views regarding CAM; (3) quality of Life; and (4) psychological and personality traits. Where possible, validated instruments were used as described below.

IBD-specific CAM use was assessed by asking subjects to rate the frequency with which they use complementary or alternative medicine to treat IBD on an ordinal Likert scale. A dichotomous variable was then generated whereby "yes" responses encompassed those describing their use as "often" or "very often", and "no" included responses "sometimes", "rarely" and "never".

Medication Adherence was assessed using the Morisky 4 item Self Report Measure of Medication Taking Behaviour^[21,22], examining predominantly dose omission, and covert dose reduction (CDR), the tendency to take less than prescribed of IBD medication without prescriber awareness was assessed as a dichotomous variable (yes/no) based on answer to the question "I take less than prescribed of my IBD medication without telling my doctor". This has been previously reported^[20].

Free text responses regarding attitudes towards IBD medication and dose modification were encouraged.

Other non-standardised attitudinal statements

were put to subjects, seeking their views regarding IBD treatment beliefs and attitudes. Some Likert data were collapsed into categories "yes" and "no" for data presentation, but analysed as ordinal data or continuous data using factor scores for regression analysis.

Anxiety and Depression were measured using the Hospital Anxiety and Depression Scale^[23], higher scores indicating higher levels of anxiety or depression. Quality of Life was measured using the reliable and valid Short Inflammatory Bowel Disease Questionnaire^[24].

The Spielberger State-Trait Personality Inventory^[25-27] was used to assess and compare depressive symptoms, anxiety, anger and curiosity between cohorts in both the immediate (state) and long term (trait or personality characteristic).

Statistical analysis

Comparisons between cohort means and medians were performed using the Kruskal Wallis test for non-normally distributed values, and two tailed *t* test or ANOVA for normally distributed values. Pearson's χ^2 or Fisher's exact test were applied as appropriate for categorical data.

Significant or trend associations at univariate level ($P < 0.10$) determined which variables were included in regression analyses, along with demographic factors.

Additional continuous variables summarising themes across the questionnaire were generated using principal component analysis for ordinal data using M Plus software (V5.2), for the purpose of data reduction. An oblique (oblimin) rotation was used of 37 of the 55 Likert scale items assessing all aspects of IBD treatment. An examination of the Kaiser-Meyer Olkin measure of sampling adequacy suggested the sample was favourable (KMO = 0.618). When loadings less than 0.4 were excluded, the analysis yielded an 8 factor solution. Scores for each of these 8 factors were normally distributed.

Binary logistic regression was used to assess predictors of CAM use as a dichotomous dependent variable, adjusting for age, gender, employment and relationship status.

A *P* value of < 0.05 was considered statistically significant. Apart from factor analysis, statistical calculations were performed using IBM SPSS Statistics for Windows, version 22, 2013 (IBM Corp). The statistical methods for this study were reviewed by Dr Reme Mountifield of Flinders Medical Centre, South Australia.

RESULTS

Demographic data

Response rates to the survey differed between cohorts, with 337/612 (55.1%) of FMC and 91/180 (50.5%) of SA private invitees participating, compared

Table 1 Demographics in contrasting inflammatory bowel disease cohorts

	FMC (<i>n</i> = 337)	Private (<i>n</i> = 91)	Darwin (<i>n</i> = 35)	<i>P</i> value
Mean age respondents (yr)	50.3	52.2	48.4	0.35
Mean age non respondents (yr)	43.0	48.1	39.9	0.20
Female respondents	60.2%	60.4%	60%	0.99
Female non respondents	55.7%	52.4%	40.7%	0.07
Crohn's disease	55.2%	57.1%	48.6%	0.70
Indigenous subjects	0.9%	1.1%	2.9%	0.37
Current smokers	11.1%	13.6%	17.1%	0.09
Previous smokers	25.8%	25.0%	42.9%	0.09
Receiving disability support pension	1.8%	1.1%	5.7%	0.006
Employed	58.7%	56.7%	62.9%	0.19
Currently partnered	92.2	95.3	93.3	0.61

Table 2 Distribution of complementary and alternative therapy types reported by inflammatory bowel disease subjects

Primary (first mentioned) CAM type	Percentage of total CAM reported overall
Herbal products (<i>e.g.</i> , slippery elm, aloe vera juice, olive oil extract, green lipped mussel oil, other herbs)	30.50%
Probiotics	22.60%
Fish oil	12.10%
Chinese medicine	10.50%
Acupuncture, massage, magnetism	10.50%
Other (prayer, meditation, exercise, dietary supplements, hypnotherapy)	13.70%

CAM: Complementary and alternative therapy.

with 35/100 (35%) in Darwin ($P < 0.0001$). Non respondents did not differ from respondents by gender ($P = 0.2$), but there was a trend toward non respondents being younger than respondents (mean age 43.7 vs 50.3 years, $P = 0.065$). Darwin subjects were more likely be current or previous smokers, and to receive a disability support pension. This population has been previously reported^[20]. Demographic data are summarised in Table 1.

Frequency, demographic and clinical associations of regular CAM use

Many subjects (45.4% overall) reported regular use of CAM, with no significant difference in usage frequency between cohorts ($P = 0.594$) (Figure 1). Distribution of CAM type used is presented in Table 2, and was not significantly different between cohorts ($P = 0.626$). The regular use of more than one CAM type (*i.e.*, physical as well as homeopathic methods) was reported by 64.5% of subjects.

Rates of CAM use were higher amongst younger (46.69 vs 53.41 years, $P < 0.001$), female (52.0% vs 35.5%, $P < 0.001$), and permanently employed (51.1% vs 37.4%, $P = 0.004$) subjects. However, CAM usage did not differ by disease type ($P = 0.394$), conventional medication pill burden ($P = 0.784$), smoking status (P

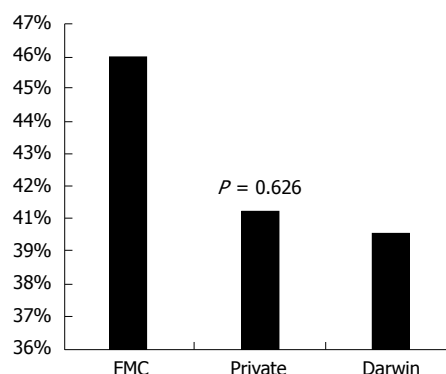


Figure 1 Proportion of subjects in each cohort reporting regular complementary medicine use.

= 0.805), or vegetarianism ($P = 0.256$) on univariate analysis.

Attitudes towards CAM

Of the 206 subjects who reported regular CAM use, 52.5% felt that it was effective (worked "well" or "very well"), and 20.7% had obtained the therapy at consultation with an alternative practitioner rather than independently. The vast majority (83.3%) continued to use conventional IBD medications concurrently. Only half (54.1%) discussed their CAM treatment with their doctor, despite 87.6% of subjects reporting feeling comfortable doing so.

In contrast, of those reporting previous consultation with an alternative practitioner only 62.2% felt comfortable discussing conventional therapy with their alternative practitioner ($P < 0.001$), and 16.6% reported the CAM practitioner discouraged their use of conventional IBD medication. With regard to the consultation experience, 10.5% felt less intimidated by alternative practitioners than doctors, and 16.9% felt more informed about IBD by the alternative practitioner.

Reasons for CAM use by free text response

Of the 194/206 (94.2%) subjects who offered reasons for their CAM use, 33.0% reported safety concerns regarding conventional medications. Subjects who elaborated further expressed the belief that "natural" CAM would enable them to reduce reliance on "chemical" conventional therapy and dose reduce or cease these medications. Seeking a holistic approach to health in some way was cited by 32.0%, and 20.6% report advice from family, friends, colleagues, religious advisors, or the internet as their main reason for use. A smaller proportion (14.4%) cited lack of efficacy of conventional medications in treating IBD. No significant cohort based differences were observed.

CAM use and treatment attitude associations-univariate analysis

Attitudinal and behavioural associations of CAM use on univariate analysis are presented in Table 3.

Table 3 Attitudinal and behavioural associations of regular complementary and alternative therapy use - univariate analysis *n* (%)

		Regular CAM use		<i>P</i> value
		No	Yes	
Deliberate dose reduction	No	197 (61.4)	124 (38.6)	< 0.001
	Yes	46 (38.7)	73 (61.3)	
Family or friends use alternative treatments	No	88 (55.0)	72 (45.0)	0.004
	Yes	85 (40.1)	127 (59.9)	
Experienced adverse effects conventional IBD meds	No	89 (59.7)	60 (40.3)	0.025
	Yes	129 (48.3)	138 (51.7)	
Satisfied with communication with IBD doctor	No	1 (9.1)	10 (90.9)	0.002
	Yes	246 (55.7)	196 (44.3)	
Previous psychological counselling	No	197 (61.6)	123 (38.4)	< 0.001
	Yes	49 (38.0)	80 (62.0)	

CAM: Complementary and alternative therapy; IBD: Inflammatory bowel disease.

Table 4 Anxiety, depression, quality of life and personality traits in users vs non users of cam in inflammatory bowel disease - univariate analysis

	Regular CAM use	Mean	SD	SE	2 tailed <i>P</i> value
Anxiety (HADS)	No	8.3312	3.50750	0.09032	0.017
	Yes	8.6365	3.18002	0.08969	
Depression (HADS)	No	6.8774	2.85105	0.07354	0.002
	Yes	6.5556	2.67318	0.07540	
SIBDQ	No	56.0152	9.71282	0.25137	< 0.001
	Yes	58.1210	9.57504	0.27126	
Trait anxiety	No	21.0042	2.53088	0.06539	0.341
	Yes	21.0957	2.48538	0.07019	
Trait curiosity	No	25.831	6.13307	0.15836	0.916
	Yes	25.8549	5.71720	0.16158	
Trait anger	No	11.3837	3.93971	0.10169	0.385
	Yes	11.5097	3.60996	0.10202	
Trait depression	No	18.9960	3.59568	0.09293	0.744
	Yes	19.0385	3.12376	0.08818	

Of all subjects including CAM users and non-users, 57.3% reported family or friends using CAM for any health purpose. Those with CAM-using contacts was more likely to use it themselves for IBD (59.9% vs 40.1%, $P = 0.004$), free text responses suggesting that type of CAM chosen was influenced by social contacts.

The 54.9% of subjects reporting adverse effects of conventional medications were more likely to use CAM ($P = 0.025$), as were the 26.9% reporting regular self-initiated dose reduction of medication ($P < 0.001$). Lack of doctor communication satisfaction was reported by only a small proportion of patients (2.4%) but was associated with CAM use, as was seeking of psychological or psychiatric treatment ($P < 0.001$) when analysed as individual items.

Analysis of HADS, QOL and Spielberger mean scores suggested that increased anxiety, higher quality of life and lower depression scores were associated with increased CAM use, whilst personality type did not

Table 5 Independent attitudinal predictors of regular complementary and alternative therapy use in inflammatory bowel disease - logistic regression analysis

	Odds ratio	95%CI	<i>P</i> value
Covert dose reduction	2.588	2.135-3.138	< 0.001
Seeking psychological treatment	1.888	1.563-2.280	< 0.001
Family and friends are regular CAM users	1.710	1.434-2.044	< 0.001
Dissatisfied with doctor communication	1.561	1.304-1.869	< 0.001
Adverse effects conventional medications	1.208	1.006-1.467	0.043
Depression (HADS)	0.910	0.878-0.943	< 0.001
Quality of life (SIBDQ)	1.022	1.011-1.032	< 0.001

CAM: Complementary and alternative therapy.

influence rate of use (Table 4).

Independent predictors of regular CAM use

After adjustment for age, gender, disease type and employment level, attitudinal and psychological predictors of regular CAM use using binary logistic regression analysis are shown in Table 5. This model explained a significant proportion of variance in low adherence rates (adjusted pseudo R squared 0.217, goodness of fit Hosmer Lemeshow $P = 0.161$).

After adjustment for demographics a trend was observed toward higher CAM usage amongst non-smokers (OR = 1.299, 95%CI: 0.993-1.698, $P = 0.056$).

Covert dose reduction, lower depression scores and subjects' propensity to seek psychological help predicted CAM use, the latter factor analysis generated variable encompassing use of antidepressants, and consultations with counsellors, psychologists or psychiatrists (Table 5). Similarly, the factor analysis generated variable assessing dissatisfaction with doctor communication was an independent predictor of CAM use, and included satisfaction level with doctor relationship, doctor communication style, level of comfort in asking questions of doctor, and comprehension of information provided during consultation.

DISCUSSION

This study demonstrates the high frequency of CAM use amongst IBD patients in Australia, and suggests that such use occurs independently of health care setting and geography. Newly identified attitudinal and psychological risk factors include dissatisfaction with patient-doctor communication, CAM use by social contacts and lower depression scores. We confirm both the known demographic risk factors for CAM use and known behavioural associations such as covert dose reduction, psychotherapeutic support seeking, and adverse effects of conventional medications.

The frequency of regular CAM use was slightly

higher in our study population (45.4%) than reported previously in Australia^[4], but within the range reported internationally^[1,4,28]. Similarly to the Italian study assessing regional variation in CAM use^[14], we found no difference in overall rates of CAM use between cohorts, but in contrast did not find regional variation in the type of CAM chosen either. Some variation in choice of CAM type is seen between populations globally, our predominantly Caucasian cohorts being comparable with New Zealand IBD subjects amongst whom herbs and vitamins were most commonly used^[5]. Interestingly nearly two thirds of subjects used more than one type of CAM, however, overlapping physical and homeopathic methods and rendering further analysis by individual CAM type difficult.

Although the patient doctor relationship is known to affect CAM use^[29], the more specific aspect of doctor communication quality as a predictor has not been previously reported. Subjects who were dissatisfied with the style of communication from their doctor, did not feel information was presented in a comprehensible way, or felt that the consultation environment did not encourage patient questions, were significantly more likely to use CAM after adjustment for other factors. A Canadian study found that the wish for a more active role in treatment decisions was associated with CAM use^[17], and the desire for more information from doctors was predictive of use in an Italian cohort^[30].

The significant influence of CAM use behaviours amongst social contacts on CAM uptake decisions in IBD individuals has also not been previously reported. In our study this was adjusted for age, gender, and employment level but not for other demographics which may be common across family members and confound the association. Such influence would not be surprising, however, given the effect of marital status, for example, on other medication taking behaviours such as adherence to conventional therapy in IBD^[31]. A study of healthy adolescents found that social contacts exert significant influence over the decision to use CAM^[32], and further work to investigate this in IBD populations is warranted, especially given the escalating influence of social media on everyday decision making.

Previously reported predictors including CDR of conventional medications, adverse effects of medications and increased QOL were confirmed in this study. Free text responses strongly suggested that IBD CAM users tend to reduce rather than omit doses of conventional medications on the assumption that CAM use will provide a "medication sparing" effect, the aim being to minimise adverse effects of conventional medications. This newly described phenomenon is the subject of a separate publication^[20], which suggests that similar underlying health beliefs and desires drive both CAM uptake and CDR behaviour. Although abundant free text data from this study support this hypothesis, formal path analysis has yet to be

undertaken to confirm the direction of causality in the association between CAM use and CDR.

Those subjects seeking psychological input such as counselling, psychologist or psychiatrist review, or antidepressant medication were significantly more likely to use CAM in this study, and this has been previously demonstrated in two European studies^[1,13]. Free text responses suggested that CAM was not being prescribed by the psychological care provider, but rather both behaviours were the result of a desire for a holistic health approach with active ways of coping, and this has been previously reported^[13]. This may be supported by our new finding that lower depression scores were associated with CAM use, perhaps indicating the presence of successfully treated depression in this population who may be more receptive to psychology.

Gastroenterologist awareness of CAM use was similar in our study to the 46% seen in a French web based study of IBD patients^[10], but greater than that found elsewhere^[3,8,33]. This communication gap may be contributed to by both consultation participants, a study examining CAM use in IBD patients from the physician perspective finding that only 8% of IBD physicians had initiated CAM conversations themselves, and only around 50% were comfortable discussing CAM with their patients^[33].

The confirmation of previously reported demographic and attitudinal CAM predictors suggests that our study population is similar to others, and thus the results generalizable to some extent. The limitations of this study include the small amount of clinical information obtainable from subjects by self-report, including disease activity and response to conventional therapy. Additionally, comparisons between cohorts were hampered by the uneven group sizes and response rates across different treatment settings. Statistical analysis differentiating by CAM type is likely to be important but was not feasible in this study as most subjects (64.5%) reported using more than one therapy type. Also, the definition of CAM is not uniform across studies and in this case was defined as what subjects felt was outside of "conventional" therapy.

CAM use is highly prevalent and appears independent of care setting and geography in IBD, and its importance to patients is often under-recognised by physicians. The quality of patient doctor communication is a key determinant, and failure to actively address CAM use in consultation may promote patient "default" to other advice sources such as family, friends and other social contacts, which ultimately undermines the patient doctor relationship.

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COMMENTS

Background

Complementary and alternative medicine (CAM) use is common in inflammatory bowel disease (IBD), and some demographic and clinical predictors have been established. This article reports a cross sectional survey of Australian subjects from contrasting care settings with IBD, focussing on the frequency and type of CAM use, and its behavioural and attitudinal predictors.

Research frontiers

In the world of ever increasing influence on everyday health decisions from social contacts *via* social media and the internet, physician understanding of patient context needs to evolve to promote strong and open partnerships with patients in making treatment decisions.

Innovations and breakthroughs

This study demonstrates that patients with IBD make decisions regarding CAM use that are subject to multiple inputs, only one of those inputs being the treating physician. The importance of unqualified health advice from social contacts needs to be acknowledged and addressed in order to optimise adherence to conventional therapy.

Applications

IBD physicians need to attend more closely than ever to clear communication with patients regarding the risks and benefits of conventional therapy, and enquire about CAM use to better understand the patient's context. Patient understanding of disease and therapy should be routinely assessed by physicians in order to correct misperceptions introduced by social contacts, alternative practitioners and the internet that may undermine successful IBD treatment.

Peer-review

This well presented study makes an important contribution to the literature as it highlights the broader context of individuals with IBD, reporting new behavioural predictors of complementary medicine uptake in this population which warrant attention during consultation.

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Randomized Controlled Trial

Comparison of the efficacy and safety of sedation between dexmedetomidine-remifentanil and propofol-remifentanil during endoscopic submucosal dissection

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Clinical trial registration: This study is registered at <http://ClinicalTrials.gov>. The registration identification number is No. NCT01920113.

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Abstract

AIM: To compare the efficacy and safety of sedation protocols for endoscopic submucosal dissection (ESD) between dexmedetomidine-remifentanil and propofol-remifentanil.

METHODS: Fifty-nine patients scheduled for ESD were randomly allocated into a dexmedetomidine-remifentanil (DR) group or a propofol-remifentanil (PR) group. To control patient anxiety, dexmedetomidine or propofol was infused to maintain a score of 4-5 on the Modified Observer's Assessment of Alertness/Sedation scale. Remifentanil was infused continuously at a rate of 6 µg/kg per hour in both groups. The ease of advancing the scope into the throat, gastric motility grading, and satisfaction of the endoscopist and patient were assessed. Hemodynamic variables and hypoxemic events were compared to evaluate patient safety.

RESULTS: Demographic data were comparable between the groups. The hemodynamic variables and pulse oximetry values were stable during the procedure in both groups despite a lower heart rate in the DR group. No oxygen desaturation events occurred in either group. Although advancing the scope into the throat was easier in the PR group ("very easy" 24.1% vs 56.7%, $P = 0.010$), gastric motility was more

suppressed in the DR group ("no + mild" 96.6% *vs* 73.3%, $P = 0.013$). The endoscopists felt that the procedure was more favorable in the DR group ("very good + good" 100% *vs* 86.7%, $P = 0.042$), whereas patient satisfaction scores were comparable between the groups. *En bloc* resection was performed 100% of the time in both groups, and the complete resection rate was 94.4% in the DR group and 100% in the PR group ($P = 0.477$).

CONCLUSION: The efficacy and safety of dexmedetomidine and remifentanyl were comparable to propofol and remifentanyl during ESD. However, the endoscopists favored dexmedetomidine perhaps due to lower gastric motility.

Key words: Dexmedetomidine; Efficacy; Peristalsis; Safety; Endoscopic submucosal dissection

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Core tip: Propofol and remifentanyl are effectively used for endoscopic procedures. However, deep sedation especially with propofol is frequently associated with cardiorespiratory complications; therefore, it is of interest to identify shallower yet equally effective sedation protocols. Dexmedetomidine allows sedation without respiratory depression, and has also been utilized for sedation for endoscopic procedures. This study compared the efficacy and safety between propofol-remifentanyl and dexmedetomidine-remifentanyl during endoscopic submucosal dissection (ESD) from the perspective of the endoscopist and the patient. We found that efficacy and safety of dexmedetomidine-remifentanyl were comparable to propofol-remifentanyl during ESD, but the endoscopists favored the dexmedetomidine-remifentanyl regimen perhaps due to lower gastric motility.

Kim N, Yoo YC, Lee SK, Kim H, Ju HM, Min KT. Comparison of the efficacy and safety of sedation between dexmedetomidine-remifentanyl and propofol-remifentanyl during endoscopic submucosal dissection. *World J Gastroenterol* 2015; 21(12): 3671-3678 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3671.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3671>

INTRODUCTION

Endoscopic submucosal dissection (ESD) is associated with greater and longer patient discomfort and pain than other endoscopic procedures. Therefore, it is of interest to reduce pain and discomfort associated with ESD^[1]. Propofol has been widely used for endoscopic procedures^[2,3]. It is safe and effective^[4] and is associated with shorter recovery time and better sedation and amnesia levels without an increased

risk for cardiopulmonary complications^[5] than other traditional sedatives. However, in addition to the dose-dependent respiratory depression of propofol, aspiration pneumonia occurs with an incidence of 2.3% following ESD^[6]. Moreover, it is difficult to control sedation depth with propofol^[7]. However, its use in combination with other analgesics can offset these complications by reducing the dose of propofol^[8].

Dexmedetomidine, a selective α_2 -adrenoceptor agonist with sedative and analgesic effects, has been successfully used during colonoscopy^[9], cystoscopy^[10] and ESD^[11]. Dexmedetomidine suppresses gastrointestinal motility and inhibits gastric emptying in healthy volunteers^[12] whereas propofol does not^[13]. Suppressing gastric motility may be crucial for successful ESD.

In this study, we compared the procedural efficacy and patient safety of the use of dexmedetomidine-remifentanyl *vs* propofol-remifentanyl during ESD.

MATERIALS AND METHODS

Patient and sedation protocol

This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University Health System (ref: 4-2012-0621) and was registered at <http://ClinicalTrials.gov> (ref: NCT01920113). Written informed consent was obtained from all patients before the procedure. Sixty patients aged > 20 years belonging to American Society of Anesthesiologists classification I - III and scheduled for ESD were enrolled in this prospective, randomized, and endoscopist-blind study from September 2012 to January 2013. Patients with end-organ diseases (*i.e.*, heart failure, respiratory failure, hepatic failure, or renal failure), known drug allergies, or a history of drug abuse were excluded.

The patients were randomly assigned to the dexmedetomidine-remifentanyl group (DR group, $n = 30$) or the propofol-remifentanyl group (PR group, $n = 30$) using a random number table provided by www.random.org. Among the 60 patients, data for 59 patients (29 patients in the DR group and 30 patients in the PR group) were analysed; surgical removal was considered in one patient.

Both the endoscopists and patients were blinded to the sedation protocol. None of the patients were pre-medicated. The level of sedation in both groups was targeted to a score of 4-5 on the Modified Observer's Assessment of Alertness/Sedation scale^[14] (MOAA/S, Table 1) for minimal sedation during the entire procedure. For the DR group, a bolus dose of 0.5 $\mu\text{g/kg}$ dexmedetomidine (Precedex[®], Abbott, Istanbul, Turkey) was injected intravenously for 5 min before starting the procedure. Thereafter, a continuous infusion dose of 0.3-0.7 $\mu\text{g/kg}$ per hour was given. For the PR group, a bolus injection of 0.5 mg/kg propofol was followed by continuous infusion at a rate of 30 $\mu\text{g/kg}$ per minute (Pofol[®], Dongkook Pharm.

Table 1 Modified observer's assessment of alertness/sedation

Alertness/sedation level	Description
6	Agitated
5	Respond readily to name spoken in normal tone (alert)
4	Lethargic response to name spoken in normal tone
3	Responds only after name is called loudly, repeatedly, or both
2	Responds only after mild prodding or shaking
1	Does not respond to mild prodding or shaking
0	Does not respond to deep stimulus (asleep)

Table 2 Modified aldrete scoring system

Discharge criteria	Score
Activity: Able to move voluntarily or on command	
Four extremities	2
Two extremities	1
Zero extremities	0
Respiration	
Able to deep breathe and cough freely	2
Dyspnea, shallow or limited breathing	1
Apneic	0
Circulation	
Blood pressure \pm 20 mmHg of preanesthetic level	2
Blood pressure \pm 20 - 50 mmHg preanesthetic level	1
Blood pressure \pm 50 mmHg of preanesthetic level	0
Consciousness	
Fully awake	2
Arousable on calling	1
Not responding	0
O ₂ saturation	
Able to maintain O ₂ saturation > 92% on room air	2
Needs O ₂ inhalation to maintain O ₂ saturation > 90%	1
O ₂ saturation < 90% even with O ₂ supplementation	0

Co. Ltd., Seoul, South Korea) using an infusion pump (Syringe Pump TE-331, Terumo, Tokyo, Japan). In both groups, remifentanyl (Ultiva®, GlaxoSmithKline, Co. Ltd., Genval, Belgium) was infused continuously at the rate of 6 μ g/kg per hour beginning 5 min before commencing the procedure.

We monitored the MOAA/S scale score continuously. If the score was 6 or the patient wanted deeper sedation, a bolus of 10 mg propofol was administered. If the patient complained of pain during the procedure, 0.1 μ g/kg remifentanyl bolus was administered, and its infusion rate was increased by 0.1 μ g/kg per hour.

Hartman's solution was administered at a rate of 3-5 mL/kg per hour, and 2 L/min oxygen was given through a nasal cannula. Oxygen saturation (SpO₂), systolic and diastolic blood pressure (SBP and DBP), electrocardiogram (ECG), and heart rate (HR) were monitored continuously and recorded at 5-min intervals.

The MOAA/S scale score was recorded as follows: just before the procedure (baseline, T0); 1 min after induction of sedation (1 min after a 5 min loading of dexmedetomidine in the DR group and 1 min after the propofol bolus injection in the PR group, T1); as

Table 3 Evaluation of gastric motility

Grade of gastric motility
No
No or very weak gating movement of the pyloric ring is observed, but the movement does not show strong contraction → No peristalsis
Mild
A circular peristaltic wave is formed in the antrum but disappears without reaching the pyloric ring, or circular contraction temporarily occurs immediately before the pyloric ring → Peristaltic wave does not reach the pyloric ring
Moderate
A pronounced peristaltic wave is formed and reaches the pyloric ring → Peristaltic wave reached the pyloric ring, which opens and closes, showing star-like contraction as a result of the peristaltic wave
Vigorous
Peristaltic wave is deep and pronounced and proceeds, strangulating the antrum → Peristaltic wave reaches the pyloric ring, and the pyloric ring is totally covered by the wave, the area exhibiting star-like contraction protrudes toward the opening of the pyloric ring, and the mucosa is pushed out from the central part of the opening

the endoscope was passed into the esophagus (T2); as the tumor margin was marked by argon plasma coagulation (T3); 5 min after an injection of normal saline containing epinephrine (0.01 mg/mL) was given in the gastric submucosa (T4); at dissection of the gastric tumor region from the gastric submucosa (T5); once bleeding control was performed at the gastric bed after dissection (T6); and at the end of the procedure (T7).

The discharge Aldrete score^[15] (Table 2) was recorded to document the patient's general status at the end of the procedure.

All patients were observed in the post-anesthetic care unit (PACU) until their discharge Aldrete score reached 10.

Assessment of the efficacy of procedural performance

The ease of advancing the scope through the throat (four grades: very easy, easy, slight difficulty, and difficult), gastric motility^[16] (four grades: no, mild, moderate, and vigorous) (Table 3), and procedural satisfaction (four grades: very good, good, fair, and bad) were evaluated by the endoscopists. Gastric motility was assessed at the time after the scope had reached to stomach. Thereafter butylscopolamine (20 mg) was administered to suppress gastric motility during the procedure at the request of the endoscopists. The total amount of butylscopolamine used was recorded.

The rate of *en bloc* resection and complete resection (defined as *en bloc* resection with tumor-free margins)^[17] was compared between the groups.

Patients were also asked about their satisfaction with the procedure (four grades: very good, good, bearable, and unbearable) before discharge from the PACU.

Table 4 Patient characteristics

	DR group (n = 29)	PR group (n = 30)	P value
Age (yr)	62.1 ± 10.3	62.9 ± 12.3	0.763
Male	19 (65.5)	22 (73.3)	0.514
Height (cm)	162.2 ± 7.7	164.8 ± 5.8	0.274
Weight (kg)	62.8 ± 8.5	65.1 ± 10.2	0.276
ASA classification n (%)			0.390
I	19 (65.5)	15 (50.0)	
II	9 (31.0)	12 (40.0)	
III	1 (3.4)	3 (10.0)	
Snoring history	9 (31.0)	7 (23.3)	0.506

Values are presented as the mean ± SD or frequency (percentage). DR group: Indicates dexmedetomidine-remifentanyl group; PR group: Propofol-remifentanyl group; ASA: American society of anesthesiologists.

Table 5 Tumor characteristics n (%)

		DR group (n = 29)	PR group (n = 30)	P value
Number of lesion		36	32	
Histology	Adenoma	19 (52.8)	17 (53.1)	0.995
	Carcinoma	16 (44.4)	14 (43.8)	
	Others	1 (2.8)	1 (3.1)	
Macroscopic appearance	Elevated	32 (88.9)	27 (84.4)	0.584
	Flat or depressed	4 (11.1)	5 (15.6)	
Location	Upper body	3 (8.3)	3 (9.4)	0.945
	Middle body	8 (22.2)	8 (25.0)	
	Lower body	25 (69.4)	21 (65.6)	
Size (mm)		15.7 ± 7.0	14.0 ± 6.7	0.344

Values are presented as the mean ± SD or frequency (percentage).

Assessment of patient safety

Hemodynamic variables of SBP, DBP, HR, and SpO₂ were compared when measuring the MOAA/S score.

All respiratory (apnea and oxygen desaturation) and hemodynamic (hypertension, hypotension, tachycardia, or bradycardia; defined as a change in baseline value of more than 20%) adverse events were recorded. Apnea was defined as not breathing spontaneously for at least 20 s. Oxygen desaturation was defined as SpO₂ < 90%. We managed adverse respiratory events with a jaw thrust, mask ventilation, or by increasing oxygen flow. Ephedrine, nicardipine, atropine, or esmolol was administered for adverse hemodynamic events. The total amount of sedative drug and remifentanyl were recorded.

Statistical analysis

The statistical methods of this study were reviewed by statisticians (Mi Kyung Song and Bo Gyoung Ma) from Biostatistics Collaboration Unit, Yonsei University College of Medicine, Seoul, South Korea. Data on baseline characteristics of study participants were presented as mean ± SD for continuous variables or frequency (percentage) for categorical variables.

Table 6 Drugs used for endoscopic submucosal dissection

	DR group (n = 29)	PR group (n = 30)	P value
Sedation duration (min)	42.8 ± 26.7	37.6 ± 18.5	0.477
Dexmedetomidine infusion rate (µg/kg per hour)	0.5 ± 0.3		
Propofol infusion rate (µg/kg per minute)		23.8 ± 16.5	
Remifentanyl infusion rate (µg/kg per hour)	5.7 ± 1.4	6.3 ± 4.0	0.451
Additional propofol required			
Patients	8 (27.6)	3 (10.0)	0.083
Dose (mg)	16.9 ± 10.3	13.3 ± 5.8	0.596
Butylscopolamine use			
Patients	4 (13.8)	10 (33.3)	0.078
Dose (mg)	3.4 ± 9.3	10.0 ± 16.4	0.066

Values are presented as the mean ± SD or frequency (percentage).

Continuous and categorical variables were tested by using Student's *t* test and χ^2 test (or Fisher's exact test), respectively. Repeatedly measured variables such as SpO₂, SBP, DBP, and HR were analyzed by a linear mixed model with patient indicator, group, time, and interaction between group and time as fixed effect factors. When the interaction between group and time was significant, post-hoc testing was performed with Bonferroni correction. All statistical tests were two-tailed at a significance level of 0.05. Statistical analyses were performed by using SPSS software (ver. 19.0, SPSS Inc., Chicago, IL, United States) and PASS software (ver. 12, NCSS, LLC, Kaysville, Utah, United States).

The sample size of this study was referred from the previous randomized trial^[18] comparing the safety and effectiveness between dexmedetomidine and propofol during oesophagus interventions.

RESULTS

No significant differences were observed in patient demographic data including age, sex ratio, height, weight, snoring history, and ASA classification (Table 4). Tumor characteristics, including histology, macroscopic appearance, location and size measured by the endoscopist were similar between the groups (Table 5).

Dexmedetomidine in the DR group and propofol in the PR group were infused at rates of 0.5 ± 0.3 µg/kg per hour and 23.8 ± 16.5 µg/kg per minute, respectively. The infusion rates of remifentanyl were 5.7 ± 1.4 µg/kg per hour and 6.3 ± 4.0 µg/kg per hour in the DR and PR groups, respectively (*P* = 0.451). Eight and 3 patients in the DR and PR groups, respectively, required propofol as a rescue sedative (*P* = 0.083) at 16.9 ± 10.3 mg and 13.3 ± 5.8 mg (*P* = 0.596), respectively (Table 6).

Complete resection was possible with 94.4% of the 36 *en bloc* resections in the DR group and 100.0% of the 32 *en bloc* resections in the PR group. Moreover,

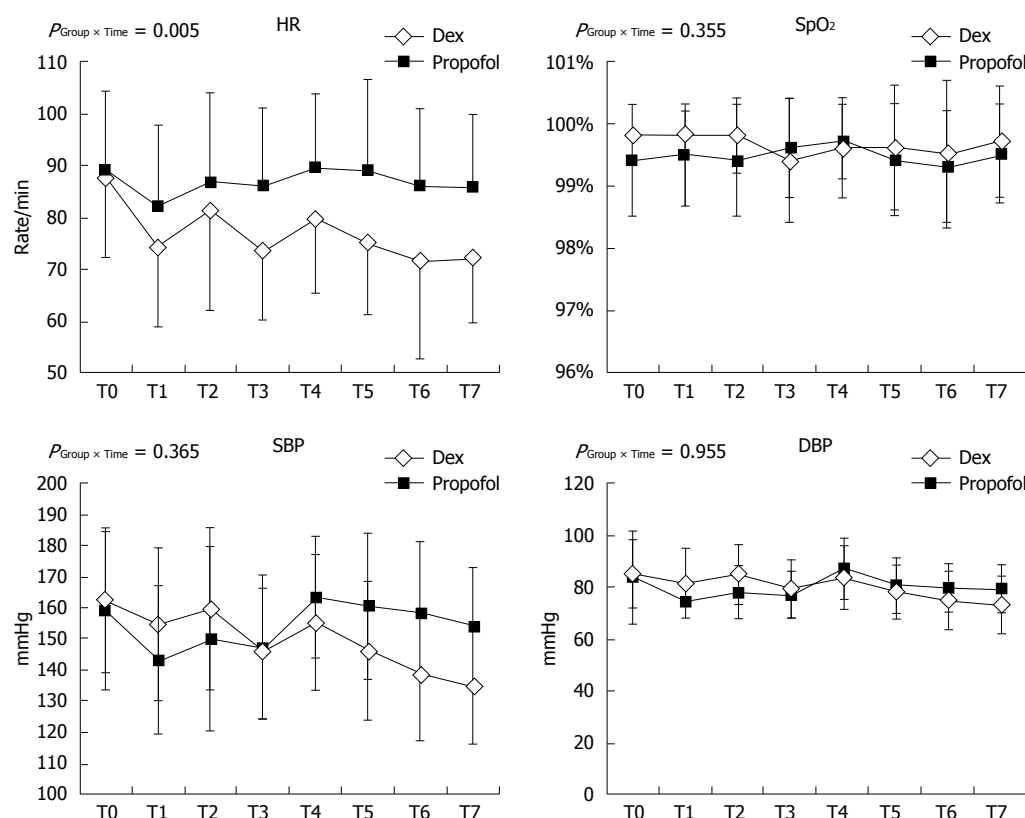


Figure 1 Changes of hemodynamic variables and SpO₂ during endoscopic submucosal dissection. T0, just before the procedure; T1, 1 min after induction of sedation (1 min after a 5 min loading of dexmedetomidine in the DR group and 1 min after the propofol bolus injection in the PR group); T2, as the endoscope was passed into the esophagus; T3, as the endoscope marked the tumor region; T4, 5 min after epinephrine injection was given in the gastric submucosa; T5, at dissection of the gastric tumor region from the gastric submucosa; T6, once bleeding control was reached at the gastric bed; T7, and at the end of the procedure. HR: Heart rate; SpO₂: Oxygen saturation; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

the duration of sedation was similar ($P = 0.477$).

Although the endoscope was more easily advanced through the throat in the PR group than in the DR group ($P = 0.010$), low-grade gastric motility (no or mild) was more frequent in the DR group (96.6% vs 73.3%, $P = 0.013$). Butylscopolamine was administered to 10 patients of the PR group compared with 4 patients of the DR group ($P = 0.078$).

While the endoscopists were satisfied with the procedural performance and judged the procedures as favorable in all patients in the DR group and in only 86.7% of patients in the PR group ($P = 0.042$), patient satisfaction was comparable between the two groups (Table 7).

The Aldrete score at the end of the procedure was not different between the groups (9.5 ± 0.6 in the DR group and 9.4 ± 0.6 in the PR group, $P = 0.924$) and all patients left the PACU within 30 min (21.2 ± 6.8 min in the DR group and 20.4 ± 5.8 min in the PR group, $P = 0.636$).

No differences in the MOAA/S scale score, SBP, DBP, or SpO₂ were observed. However, the mean change of HR over time was different between DR and PR groups (Figure 1). No cases of oxygen desaturation or any adverse hemodynamic events were observed during the ESD procedures in either group.

DISCUSSION

We found that minimal sedation using dexmedetomidine-remifentanyl could be substituted for propofol-remifentanyl during ESD, and that the endoscopists seemed to be satisfied with the procedural efficacy perhaps due to low gastric motility.

This study has some clinical implications regarding the sedating protocol for ESD. First, our results suggest the importance of analgesics and optimal sedation level to avoid patient anxiety. ESD was safely performed under MOAA/S sedation levels of 4-5 if adequate analgesic was provided. As shown in Figure 1, no patient needed management due to hemodynamic instability or adverse respiratory events despite the decreased HR in the DR group. We believe that continuous infusion of remifentanyl enabled the patients to tolerate this procedure well in an orientated and anxiety-free state. The analgesic requirement for a painful procedure was evident in a previous colonoscopy trial, which was terminated early before enrolling the planned number of patients because of the higher rate of supplemental fentanyl required and adverse hemodynamic events in the group of patients administered dexmedetomidine alone^[19]. International sedation guidelines for gastrointestinal

Table 7 Efficacy of procedural performance

	DR group (n = 29)	PR group (n = 30)	P value
Advancing scope into throat			0.010
Very easy	7 (24.1)	17 (56.7)	
Easy	14 (48.3)	12 (40.0)	
Slight difficult	1 (3.4)	1 (3.3)	
Difficult	7 (24.1)	0 (0.0)	
Gastric motility			0.101
No	21 (72.4)	16 (53.3)	
Mild	7 (24.1)	6 (20.0)	
Moderate	1 (3.4)	7 (23.3)	
Vigorous	0 (0.0)	1 (3.3)	
Low: No + mild	28 (96.6)	22 (73.3)	0.013
High: Moderate + vigorous	1 (3.4)	8 (26.7)	
Endoscopist's satisfaction			0.216
Very good	21 (72.4)	17 (56.7)	
Good	8 (27.6)	9 (30.0)	
Fair	0 (0.0)	2 (6.7)	
Bad	0 (0.0)	2 (6.7)	
Favorable: Very good + good	29 (100.0)	26 (86.7)	0.042
Unfavorable: Fair + bad	0 (0.0)	4 (13.3)	
Patients' satisfaction of sedation			0.616
Very good	4 (13.8)	7 (23.3)	
Good	21 (72.4)	20 (66.7)	
Bearable	4 (13.8)	3 (10.0)	
Unbearable	0 (0.0)	0 (0.0)	

Values are presented as frequency (percentage). The gastric motility and endoscopists' satisfaction were reclassified as low (no + mild) or high (moderate + vigorous) and favorable (very good + good) or unfavorable (fair + bad), respectively.

endoscopic procedures^[20-22] recommend sedating patients to improve procedural performance. However, the adequate level of sedation for patients has not been well defined (conscious sedation vs deep sedation). Takimoto *et al.*^[11] compared the efficacy and safety of conscious sedation for ESD targeting a Ramsay sedation score (RSS) of 2-3 among propofol, dexmedetomidine, and midazolam. They found that dexmedetomidine provided comparable hemodynamic stability and improved oxygen saturation as well as no major surgical complications compared to propofol or midazolam. In comparison, two patients who received propofol or midazolam developed gastric perforation. An RSS of 2-3 represents a level of sedation that is similar to, but slightly deeper than, the MOAA/S of 4-5 used in the present study (MOAA/S 4 = responding to normal verbal tone; RSS 3 = responding to commands). Sasaki *et al.*^[19] reported hypoxemia in 15.9%-17.8% of patients and hypotension in 19.3%-34.4% of patients, suggesting a deeper sedation level and a higher rate of complications. In the present study, minimal sedation, regardless of the group, allowed the patients to achieve an Aldrete score of 9.5 at the end of the procedure and to leave the PACU within 30 min. Fast recovery may also be an economic benefit of minimal sedation. However, further pharmaco-economic evaluation between propofol and dexmedetomidine will not be discussed here because of the costal differences of medications

among countries.

Second, regarding procedural performance, the endoscopists felt that the endoscope could be more easily advanced into the throat with propofol (endoscopists reported the insertion "very easy" in 7 of 29 patients in the DR group vs 17 of 30 patients in the PR group, $P = 0.01$). The underlying causes of this difference are unclear but might be explained, in part, by the different effect of propofol and dexmedetomidine on the pharyngeal function. Kiriya *et al.*^[23] assessed the effects of a bolus of 0.5 mg/kg propofol injected before ESD compared to no bolus of propofol and found that the propofol bolus decreased pharyngeal muscle tone and obtunded the scope-stimulated pharyngeal reflex in 77% of patients compared to 21% of patients with no bolus. Therefore, in the present study, the intact pharyngeal function in the DR group may have made it more difficult for the endoscopists to advance the scope into the throat.

Inhibiting gastric motility is crucial for successful performance of ESD, and this is the first report of endoscopist evaluated gastric motility during ESD in relation to two different sedation protocols (Table 7). The endoscopists graded gastric motility as low (no and mild among four grades) in 96.6% of the DR group and in 73.3% of the PR group ($P = 0.013$). Corroborating the report of lower gastric motility by the endoscopists, patients in the DR group required butylscopolamine less frequently to suppress gastric motility than those in the PR group. The effects of dexmedetomidine on gastric motility seemed to differ according to subject and dosage. In a previous study, infusion with a 1.0 $\mu\text{g/kg}$ loading dose for 20 min followed by infusion of 0.7 $\mu\text{g/kg}$ per hour inhibited gastric emptying in healthy volunteers, as measured by paracetamol absorption compared to 0.1 mg/kg morphine or placebo^[12]. In contrast, Memiş *et al.*^[24] found no difference in gastric emptying time between propofol (2 mg/kg per hour) and dexmedetomidine (0.2 $\mu\text{g/kg}$ per hour) for 5 h in critically ill patients. This discrepancy may have resulted from the different doses of drugs and measuring methods (direct visualization vs indirect paracetamol absorption test) used in the two studies. Dexmedetomidine itself does not alter gastric motility in rats but markedly enhances the inhibitory effect of morphine on gastric motility^[25]. We are uncertain of the interactive effect of dexmedetomidine and remifentanyl on gastric motility. We believed that both sedation protocols were effective for ESD considering that the endoscopists were able to perform complete resection at a comparable rate between the two groups (94.4% vs 100%).

However, our study had some limitations. We analyzed a small number of patients, which limited the statistical power of our results. If we carry out the follow-up study, 143 subjects will be needed for each group to keep the statistical power of 80%. Gastric motility did not differ between the two groups ($P = 0.101$) when measured using the four grades (no,

mild, moderate, and vigorous); however, there was a significant difference when just two grades of low (no/mild) and high (moderate/vigorous) were applied ($P = 0.013$). This same issue was also observed with the statistical analysis of endoscopists' satisfaction. We did not find any significant difference when the ratings were based on four grades (very good, good, fair, and bad). However, when satisfaction was divided into favorable (very good/good) and unfavorable (fair/bad), the endoscopists were in favor of the dexmedetomidine-remifentanyl treatment (favorable, 100% in the DR group vs 86.7% in the PR group, $P = 0.042$). Although there were no serious adverse respiratory events during ESD in both groups, we could not exclude the possibility of hypercapnia because we did not measure the partial pressure of carbon dioxide (PaCO_2) through arterial blood gas analysis. To blind endoscopists from the type of anesthesia, we covered the patients' venous access sites with a drape; however, we are unsure whether endoscopists were able to correctly identify the type of sedative drugs from the difference in the pharmacologic properties between dexmedetomidine and propofol. Therefore, we could not completely eliminate the bias of personal preference when they answered the questionnaires. Finally, our study design did not include a psychometric test for patients or comprehensive questionnaires to assess patients and endoscopists satisfaction as suggested by Vargo *et al*.^[26]

In conclusion, use of dexmedetomidine and remifentanyl targeting minimal sedation could be substituted for propofol-remifentanyl sedation during ESD procedure. However, the effect of suppressing gastric motility with dexmedetomidine-remifentanyl sedation needs further studies with a greater number of subjects.

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COMMENTS

Background

Endoscopic submucosal dissection (ESD) is accompanied with greater and longer patient discomfort and pain than conventional endoscopic procedures. Therefore, a combination regimen with a sedative and an analgesic, such as propofol and remifentanyl, is recommended. However, propofol depresses respiratory function in a dose-dependent manner and aspiration pneumonia can occur. Dexmedetomidine, another sedative agent with lack of respiratory depression, suppresses gastrointestinal motility and may improve the chances for successful ESD. Thus, we compared the procedural efficacy and patient safety of dexmedetomidine-remifentanyl to propofol-remifentanyl during ESD.

Research frontiers

Aspiration pneumonia occurs with an incidence of 2.3% following ESD under sedation with propofol perhaps due to dose-dependent respiratory depression. Dexmedetomidine, a selective α_2 -adrenoceptor agonist with sedative and analgesic effects that preserves ventilator function, has been successfully used

during colonoscopy. In addition, dexmedetomidine suppresses gastrointestinal motility in healthy volunteers whereas propofol does not.

Innovations and breakthroughs

The sedation level was maintained at MOAA/S of 4-5 with either sedating regimen (propofol-remifentanyl or dexmedetomidine-remifentanyl). What we found in this study were as follows. The patients tolerated the ESD procedure well and safely under minimal sedation under either sedating regimen with pain adequately controlled. However, the endoscopists favored the regimen of dexmedetomidine-remifentanyl perhaps due to the suppression of gastric motility.

Applications

Because the endoscopic procedure can vary greatly among patients in regard to anxiety, pain, and duration, it is important to regard sedation and analgesia separately to avoid over-sedation. Dexmedetomidine may provide benefits to the sedation regimen during ESD because it has sedative effects, analgesic effects, the ability to suppress gastric motility and, more importantly, the ability to preserve respiratory drive.

Terminology

The endoscopic submucosal dissection procedure lasts for hours and is accompanied by pain. Propofol and dexmedetomidine are commonly used sedative agents that work through GABAA receptor and a selective α_2 -adrenoceptor, respectively. Remifentanyl is a synthetic opioid with rapid onset and offset of action.

Peer-review

The authors compared the efficacy and safety of sedation between dexmedetomidine-remifentanyl and propofol-remifentanyl for use during ESD. Fifty-nine patients scheduled for ESD were randomly assigned to a dexmedetomidine-remifentanyl group or a propofol-remifentanyl group. The efficacy and safety of dexmedetomidine and remifentanyl were comparable to propofol and remifentanyl during ESD. The endoscopists favored dexmedetomidine mainly due to lower gastric motility.

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Cholecystectomy and the risk of alimentary tract cancers: A systematic review

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Abstract

AIM: To investigate the association between cholecystectomy and gastro-intestinal tract (GIT) cancers.

METHODS: We conducted a systematic review according to the PRISMA guidelines. A MEDLINE search was performed with predefined search criteria for English Language articles on the association between cholecystectomy and GIT cancers. Additional articles were retrieved by manual search of references. All relevant articles were accessed in full text. Data on

study type; cases; controls; country; effect estimate; adjustments for confounders and quality of publication were extracted. The quality of the publications were scored by adherence to the STROBE checklist. The data for each part of the GIT were presented in separate tables.

RESULTS: Seventy-five studies and 5 meta-analyses satisfied the predefined criteria for inclusion and were included in this review. There were inconsistent reports and no strong evidence of an association between cholecystectomy and cancers of the oesophagus (Adenocarcinoma), pancreas, small bowel and right-sided colon cancers. In squamous cancer of the oesophagus, cancers of the stomach, liver, bile ducts, small bowel and left sided colon cancers, good quality studies suggested a lack of association with cholecystectomy. Equally, distal colon and rectal cancers were found not to be associated with cholecystectomy. Several mechanisms for carcinogenesis/promotion of carcinogenesis have been proposed. These have focused on a role for bile salts in carcinogenesis with several potential mutagenic molecular events and gut metabolic hormones signaling cell proliferation or initiation of carcinogenesis.

CONCLUSION: This is a comprehensive review of the association between GIT cancers and cholecystectomy. This review found no clear association between cholecystectomy and GIT cancers.

Key words: Cholecystectomy; Cancer; Gastro-intestinal tract; Carcinogenesis

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Core tip: This systematic review explores the association between cholecystectomy and individual gastro-intestinal tract cancers and proposed mechanisms of carcinogenesis. The review finds no clear association

between cholecystectomy and cancers of the gastro-intestinal tract.

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INTRODUCTION

The presence of gallstones increases with age with an estimated median prevalence ranging from 5.9% to 21.9% in large population surveys^[1]. Gallstone disease constitutes a significant health problem affecting 10% to 15% of adults in the western world^[2-4]. Gallstone related problems such as cholecystitis and choledocholithiasis are becoming the leading cause of inpatient hospital admissions for gastrointestinal problems^[5]. Cholecystectomy is the treatment of choice for symptomatic cholelithiasis. Since 1988, laparoscopic cholecystectomy has evolved globally and more than 90% of cholecystectomies are carried out either acutely or electively using laparoscopy. Laparoscopic cholecystectomy has become standard practice for benign gallbladder disease^[6]. Several studies have shown an early increase of cholecystectomies after the adoption of laparoscopic cholecystectomy^[7-9]. Some studies have shown a sustained increase which is independent of total population growth^[6].

Over the past decade, a number of studies have investigated the association between cholecystectomy and/or cholelithiasis with gastro-intestinal tract (GIT) cancers. Although cholelithiasis is reported to be strongly associated with an increased risk of biliary tract cancers^[10], the association with other GIT cancers is not established. With regards to cholecystectomy, authors of meta-analysis reported that pooled results from case-control studies had shown a significant elevation of increased cancer risk after cholecystectomy but pooled results from cohort studies had not^[11]. However, cohort studies, which are less prone to bias have been less commonly undertaken. Further, the time scale between the exposure and the risk is not always reported. This is important given that GIT cancers and cholelithiasis are common and may arise independently. However, the symptoms of cancer may be misinterpreted to be symptoms of cholelithiasis.

The proposed mechanism for the increased risk of digestive tract cancers after cholecystectomy is through alteration of bile flow^[11,12], increased exposure^[13], alteration of bile salts^[14] or alterations to metabolic hormone levels^[15].

Since both cholecystectomy and a diagnosis of cancer of the gastro-intestinal tract are common^[6,16,17], the same person could encounter both within a lifetime,

by chance alone reasonably frequently. Cancers may be missed at laparoscopic cholecystectomy for gallstones^[18]. For these reasons, it is important to establish and quantify the association between cholecystectomy and gastro-intestinal tract cancer risk to aid the informed consent process. If a real relationship exists, every patient consented for cholecystectomy, should have all the established risks explained including the potential for GIT cancers.

The objective of this study is to perform a systematic review of the literature of studies to determine whether or not there is an association between cholecystectomy and the development of GIT cancers.

MATERIALS AND METHODS

Search strategy

A comprehensive literature search of MEDLINE via the online database PubMed was carried out by two observers to identify all relevant studies for inclusion in this literature review. The search criteria (MeSH headings/index terms) included (1) "cholecystectomy"; (2) "risk of cancer" and one of the following cancer subtypes; (3) "oesophageal"; (4) "gastric"; (5) "pancreatic"; (6) "bile duct"; (7) "liver"; (8) "small intestine"; and (9) "colorectal". In addition, MeSH headings, index terms of 'bile salts', 'risk of cancer' and "carcinogenesis" were used to search for the proposed mechanism of action.

Only English language articles were included in the analysis. Review articles, case reports and studies based on autopsy results were excluded. Articles, which combined the risk for cholelithiasis and cancer, were also excluded. Articles with poor study design (e.g., Inappropriate comparisons or without controlling for appropriate confounders) were excluded. No restriction was placed on the journal in which it was published, location or date of the study. Studies should report statistical ratios with 95% confidence intervals (95% CIs) or provide data to enable derivation of rate or risk ratios. Rarely, studies reporting odds ratio (OR) with 95% CIs were included if their inclusion was deemed relevant. References from included studies were searched manually to identify missing relevant publications. When data of a study group were used in multiple articles, only the most recent paper was used for this review.

Data extraction

Each study was analyzed based on type of methodology (meta-analysis; case control; cohort) and study size indicating the number of GIT cancers and control cases. The data sources were noted for both the exposure and outcome parameters. The number of years follow up as reported in the study was recorded when available and the effect estimate [relative risk (RR); hazard ratio (HR); OR; Incidence rate ratio (IRR)] with its calculated 95%CI was noted. Where risk ratios

were adjusted for age, gender and other confounding factors this was recorded. Extracted data were stratified by the site of cancer, year of publication, country where the study was undertaken and any other relevant factors.

Assessment of study quality

The quality of the different studies was measured using the STROBE (Strengthening The Reporting of Observational Studies in Epidemiology)^[19] checklist. Each item on the STROBE checklist was scored by one of the authors as follows: 0, item not reported; 1, item reported but inadequately; 2, item reported adequately. Although there are 22 items on the STROBE checklist, item number 1 was divided into 2 sections, item number 6 was divided into two sections, item number 12 was divided into 5 sections, item number 13 was divided into three sections, item number 14 was divided into two sections and item number 16 was divided into three sections. As such, the maximum score that any publication could achieve was 66. In order to be comprehensive, no minimum score was set for inclusion.

Statistical analysis

Descriptive statistics were quoted from the original source where provided. In a few circumstances, it was necessary to derive the OR and 95%CI from the data provided. Statistical analysis was done using IBM SPSS v21 (SPSS, Chicago, IL, United States).

RESULTS

Included studies

The total number of initial publications retrieved from MEDLINE for the association between cholecystectomy and GIT cancers was 1394 articles. After screening titles and abstracts, 142 were included for full text analysis. After exclusion of studies, which did not meet the selection criteria, 75 studies (cohort and case-control) describing an association between cholecystectomy and a GIT cancer site were included for data extraction. Three meta-analyses were reviewed. A flow chart of the literature search is depicted in Figure 1.

Oesophageal adenocarcinoma and squamous cell carcinoma

Two case control studies^[20,21] with 321 cases between them and one cohort study^[22] based on 91 cases, found that cholecystectomy, despite its effect on gastric juice did not appear to increase the risk of oesophageal adenocarcinoma. By contrast, two cohort studies^[23,24] based on 179 cases, found a moderate association between cholecystectomy and subsequent oesophageal adenocarcinoma, however, the absolute risk was found to be small. The results from a meta-analysis^[25] suggested that patients who had a

cholecystectomy more than 10 years previously are at an increased risk for oesophageal adenocarcinoma (SRRs = 1.26, 95%CI: 1.06-1.49). Descriptive characteristics of studies on the association between cholecystectomy and Oesophageal Adenocarcinoma are shown in Table 1.

Two case control^[20,21] and three cohort studies^[23,24,26] based on 618 cases found that cholecystectomy was not associated with an increased risk of oesophageal squamous cell carcinoma. The results from a meta-analysis, which included some of these studies, confirmed the null association (SRRs = 0.92, 95%CI: 0.80-1.06), which was independent of study location or study design^[25]. Descriptive characteristics of studies on the association between cholecystectomy and Oesophageal squamous cell carcinoma are shown in Table 2.

Gastric cancer

Two case control studies^[26,27] based on 186 cases found that cholecystectomy did not increase the risk of gastric cancer. However, one case control study^[21] and three cohort studies^[22,28,29] based on a total of 1491 cases found an increased risk of gastric cancer after a cholecystectomy. The results from a meta-analysis, which included some of these studies, found that prior cholecystectomy was not associated with the risk of gastric cancer (SRRs = 1.03, 95%CI: 0.93-1.13). Descriptive characteristics of studies on the association between cholecystectomy and gastric cancer are shown in Table 3.

Two case control^[20,21] and one cohort study^[28] based on a total of 478 cases found that prior cholecystectomy was not associated with an increased risk of gastric cardia cancer. The results from a meta-analysis^[25] which included two studies specific for gastric cardia cancer^[20,28] found that cholecystectomy was not associated with risk of gastric cardia cancer (SRRs = 0.87, 95%CI: 0.65-1.17). Descriptive characteristics of studies on the association between cholecystectomy and gastric cardia cancers are shown in Table 4.

Pancreatic cancer

There are at least 23 epidemiological studies investigating the association between cholecystectomy and pancreatic cancer (see Table 5). The results obtained from these studies are contradictory. A significantly increased risk between previous cholecystectomy and pancreatic cancer was found in four case control studies^[21,30-32] and four cohort studies^[33-36]. However, no association was found among nine case-control studies^[37-45] and six cohort studies^[22,26,29,46-48].

A meta-analysis based on 18 studies (8 cohort studies and 10 case-control studies) reporting a total of 12129 cases of pancreatic cancer found that 9 studies reported a positive (but not significant) association between previous cholecystectomy and risk of pancreatic cancer and 5 studies found a significantly

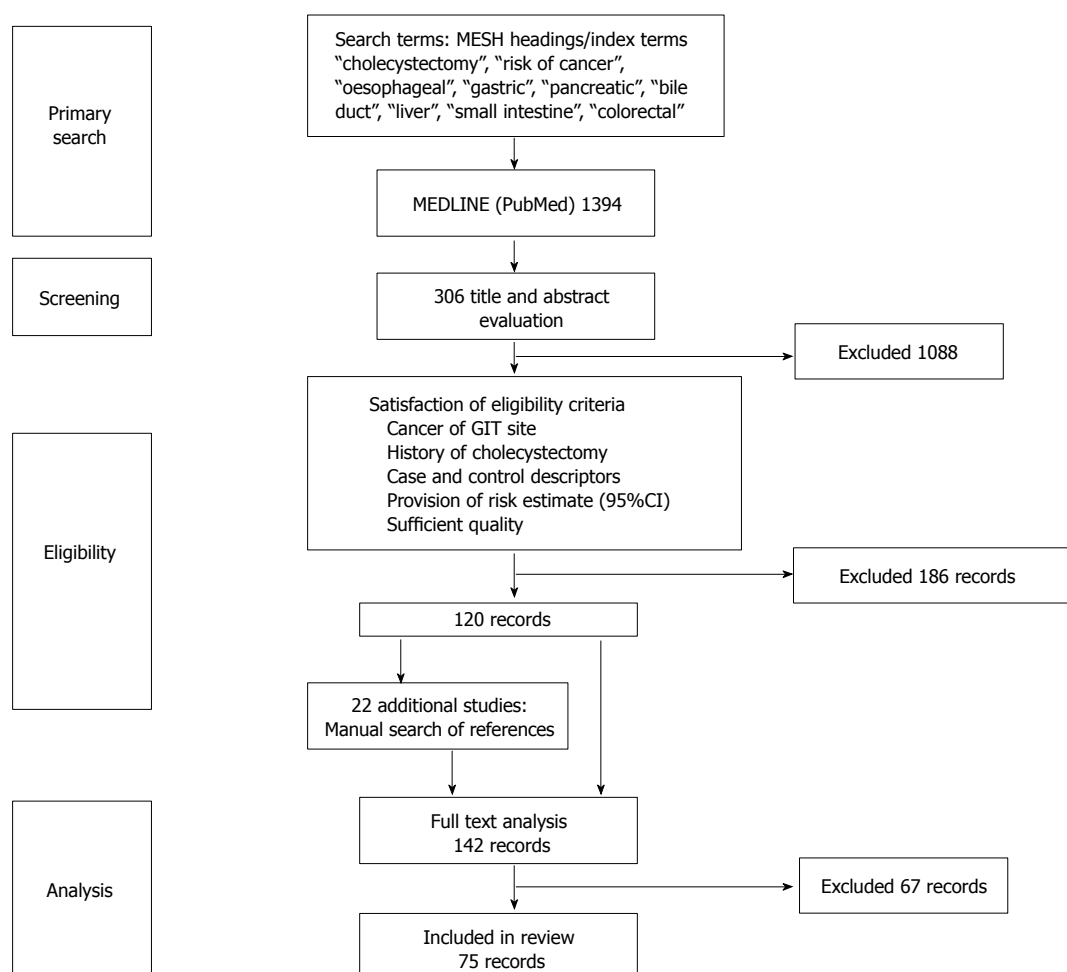


Figure 1 Study flow diagram. GIT: Gastro-intestinal tract.

increased risk of pancreatic cancer in patients who had a cholecystectomy^[49]. The meta-analysis found that compared with individuals without a history of cholecystectomy, those who had their gallbladder removed had a 23% excess risk of pancreatic adenocarcinoma (SSRs = 1.23, 95%CI: 1.12-1.35). Sub-group analysis revealed that the increased risk was independent of geographic location, gender, study design and confounders including body mass index (BMI), diabetes and smoking. The risk of pancreatic cancer remained elevated two and five years post cholecystectomy. Descriptive characteristics of studies on the association between cholecystectomy and pancreatic cancer are shown in Table 5.

Extra-hepatic bile duct cancer

A case control study comparing the incidence of cancers of the extra-hepatic bile duct and ampulla of Vater, before and after the introduction of laparoscopic cholecystectomy, found, no increase in the incidence of these cancers in the short term^[50]. The study was based on the observed increase in the rate of laparoscopic cholecystectomy since its introduction in 1990^[7-9]. One case-control study^[21] and two cohort

studies^[29,34] based on 143 cases of extra-hepatic bile duct cancer did not find a significant association in cancer risk with a history of cholecystectomy. Descriptive characteristics of studies on the association between cholecystectomy and extra-hepatic bile duct cancers are shown in Table 6.

Liver cancer

One case-control study^[21] (332 incident cases of cancer of the liver) found a significant association between a previous history of cholecystectomy and an increased risk of liver cancer (OR = 1.26, 95%CI: 1.12-1.41). This significant association was found for hepatocellular carcinoma (OR = 1.34, 95%CI: 1.17-1.52) and not for cholangiocarcinoma (OR = 1.19, 95%CI: 0.98-1.43). However, three cohort studies^[22,29,34] based on 173 incident cases of liver cancer in patients who had a previous cholecystectomy did not show an increased risk of liver cancer after cholecystectomy. Descriptive characteristics of studies on the association between cholecystectomy and liver cancer are shown in Table 7.

Intestinal (small bowel) cancer

One case control study^[21] based on 148 incident cases

Table 1 Descriptive characteristics of studies on the association between cholecystectomy and oesophageal adenocarcinoma

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Freedman <i>et al</i> ^[20] , 2000	1994-1997	Case-control	189	820	Self report	Pathology records	-	RR = 1.03 (0.63-1.69)	Age, gender, alcohol, smoking, BMI, physical activity, education, diet	37
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	132/5488	2572/1000000	Medicare database	Cancer registry	> 6	OR = 0.95 (0.80-1.14)	Age, gender, diabetes	49
Freedman <i>et al</i> ^[23] , 2001	1965-1997	Cohort	53/268312		National registry	Cancer registry	> 10	SIR = 1.3 (1.0-1.8)	Age, gender	38
Goldacre <i>et al</i> ^[22] , 2005	1963-1999	Cohort	91/39245	803/334813	NHS database	Cancer registry	NA	RR = 0.98 (0.79-1.21)	Age, gender, calendar year, residence	36
Lagergren and Mattsson ^[24] , 2011	1965-2008	Cohort	126	345251	NA	Cancer registry	15	RR = 1.29 (1.07-1.53)	Age, gender, calendar Year	40

NA: Not available; BMI: Body mass index.

Table 2 Descriptive characteristics of studies on the association between cholecystectomy and oesophageal squamous cell cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Freedman <i>et al</i> ^[20] , 2000	1994-1997	Case-control	167	820	Self-report	Pathology records		OR = 0.82 (0.43-1.54)	Age, gender, alcohol, smoking, BMI, physical activity, education, diet	37
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	100/4732	2572/100000	Medicare database	Cancer registry	> 6	OR = 0.85 (0.69-1.04)	Age, gender, diabetes	49
Ichimiya <i>et al</i> ^[26] , 1986	1953-1984	Cohort	29	1238	Self report	Death registry	< 31	0.59 (0.26-1.36)	Age, gender	48
Freedman <i>et al</i> ^[23] , 2001	1965-1997	Cohort	129/268312	NA	National registry	Cancer registry	> 10	SIR = 0.9 (0.7-1.1)	Age, gender	38
Lagergren and Mattsson ^[24] , 2011	1965-2008	Cohort	193/345251		NA	Cancer registry	15	SIR 0.93 (0.81-1.08)	Age, gender, calendar year	40

Ichimiya *et al*^[26], 1986, reported on oesophageal cancer without specifying pathology of cancer. NA: Not available; BMI: Body mass index.**Table 3** Descriptive characteristics of studies on the association between cholecystectomy and gastric cardia cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Freedman <i>et al</i> ^[20] , 2000	1994-1997	Case-control	262	820	Self-report	Pathology	-	RR = 0.67 (0.39-1.13)	Age, gender, alcohol, smoking, BMI, physical activity, education, diet	37
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	122/5579	2572/100000	Medicare database	Cancer registry	> 6	OR = 0.88 (0.73-1.06)	Age, gender, diabetes	49
Fall <i>et al</i> ^[28] , 2007	1970-1997	Cohort	94/251672	NA	National registry	Cancer registry	11.5	RR = 0.95 (0.76-1.16)	Age, gender, surgical procedure	42

NA: Not available; BMI: Body mass index.

of small bowel cancer found a significant association between a history of cholecystectomy and an increased risk of carcinoid tumors of the small bowel (OR = 1.78, 95%CI: 1.41-2.25) and a weaker increased risk of adenocarcinoma of the small bowel (OR = 1.34, 95%CI: 1.02-1.76). In addition, two cohort studies^[51,52] found a significantly elevated risk of small bowel tumors after cholecystectomy. The risk was found to be elevated for both proximal small bowel adenocarcinoma and for distal small bowel carcinoid tumors. In the first year after cholecystectomy, the age adjusted rate ratios for cancer of the small bowel were significantly high at 10.43, 95%CI: 7.79-13.99.

Thereafter, the rate ratio reduced with increasing time since operation. By 8 years and more from cholecystectomy, the rate ratio was not significantly raised at 2.47, 95%CI: 0.82-6.28^[52]. Descriptive characteristics of studies on the association between cholecystectomy and small intestine cancers are shown in Table 8.

Colorectal cancer

Three case-control studies reporting 132 cases of colorectal cancer found a significant association between cholecystectomy and colorectal cancers^[43,53,54]. The highest reported RR was 2.11 (95%CI: 1.19-3.85)^[54].

Table 4 Descriptive characteristics of studies on the association between cholecystectomy and gastric cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Sarli <i>et al</i> ^[27] , 1986	1980-1984	Case control	157	157	Surgical and database	Pathology	NA	0.77 (0.09-6.40)	Age, gender	26
Ichimiya <i>et al</i> ^[26] , 1986	1953-1984	Cohort	29	1238	Self report	Death registry	NA	0.92 (0.66-1.28)	Age, gender	33
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	429/ 12925	2572/ 100000	Medicare database	Cancer registry	> 6	OR = 1.26 (1.13-1.40)	Age, gender, diabetes	49
Goldacre <i>et al</i> ^[22] , 2005	1963-1999	Cohort	177/ 39254	1354/ 334813	NHS database	Cancer registry	NA	1.06 (0.88-1.26)	Age, gender, calendar year, residence	36
Fall <i>et al</i> ^[28] , 2007	1970-1997	Cohort	854/ 251672	NA	National registry	Cancer registry	11.5	1.11 (1.04-1.19)	Age, gender, surgical procedure	42
Chen <i>et al</i> ^[29] , 2014	2000-2010	Cohort	31/ 5850		National database	Cancer registry	10	1.81 (1.09-3.02)	Age, gender, comorbidities	53

NA: Not available.

This finding was supported by three cohort studies^[29,55,56] suggesting an increased risk of colorectal cancer by up to 56% (RR = 1.56, 95%CI: 1.12-2.17^[29]). Similar trends were identified in another four case-control and two cohort studies but these were not statistically significant^[57-62]. The largest and most recent study in the literature encompasses 3907 incident cases and, with age and gender adjustments, showed no association (OR = 0.97, 95%CI: 0.92-1.02). This finding is supported by five more studies^[63-67]. Descriptive characteristics of studies on the association between cholecystectomy and colorectal cancers are shown in Table 9.

Proximal colon cancer

Six studies (4 case-control; 2 cohort) demonstrated a positive association between proximal colon cancer and cholecystectomy^[59,62,63,67-79]. An extremely high association (OR = 5.85, 95%CI: 2.13-16.7) was found in one particular Chinese study but 95%CI were broad and a low quality assessment score indicates these findings are somewhat unreliable^[62]. However, a well-designed study scoring highly (57 out of 66) on the STROBE checklist also showed a positive association (RR = 1.35, 95%CI: 0.97-1.88). The study performed a comprehensive statistical analysis to account for several confounding factors (age, smoking, BMI, lifestyle and dietary factors, comorbid disease such as diabetes)^[59]. This strengthens the findings of the study considerably. However, this study was based on a female only cohort, which raises the possibility of increased gender-based risk. Two other studies selected for analysis showed no association between proximal colon cancers and cholecystectomy^[21,51]. Descriptive characteristics of studies on the association between cholecystectomy and colorectal cancers are shown in Table 10.

Distal colon cancer

Subgroup analysis within five of the selected studies showed that there was no association of cholecystectomies with distal colon cancer^[21,51,59,67]. However, Zeng *et al*^[62]

calculated an OR of 1.87 (95%CI: 0.943-8.14) but the design of this study and statistical methodology was poor which renders meaningful interpretation difficult. Descriptive characteristics of studies on the association between cholecystectomy and colorectal cancers are shown in Table 11.

Rectal cancer

The rectum lies farthest from the gall bladder in the GI tract and any proposed mechanism relating to altered flow of bile metabolism following cholecystectomy causing cancer would be presumed to have the least effect here. A meta-analysis of 42 studies encompassing 14226 incident cases showed no significant risk of rectal cancer following cholecystectomy (OR = 1.14, 95%CI: 0.92-1.41)^[70]. This finding is supported by three other case-control studies^[53,62,63,65] and two cohort studies^[56,64]. Linos *et al*^[57] showed a reduced risk of rectal cancer in women post cholecystectomy (RR = 0.5, 95%CI: 0.1-1.3) and an increased risk in men (RR = 2.3, 95%CI: 0.9-4.8). These findings are not clinically significant and do not correlate with any other studies. They are most likely artifact due to small sample size and lack of adjustment for confounding factors and the results should be interpreted cautiously. Descriptive characteristics of studies on the association between cholecystectomy and colorectal cancers are shown in Table 12.

Proposed mechanisms of carcinogenesis

When the normal gallbladder is *in situ*, wide physiological fluctuations occur in the bile-emptying rate from the common bile duct (CBD) into the duodenum^[71,72]. After cholecystectomy, all the bile secreted from the liver enters the CBD and drains through the sphincter of Oddi into the duodenum, thereby producing a continuous flow. Although the net effect of cholecystectomy on bile secretion is not fully understood, cholecystectomy results in globally increased trans-papillary bile flow and CBD emptying rate^[73]. The increased and continuous bile flow

Table 5 Descriptive characteristics of studies on the association between cholecystectomy and pancreatic cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Wynder <i>et al</i> ^[37] , 1973	1950-1964	Case-control	11/142	16/307	Hospital records	NA	> 2	1.57 (0.76-3.24) ¹	Age, gender, race, hospital	28
Haines <i>et al</i> ^[38] , 1982	1973-1978	Case-control	8/116	18/232	Hospital records	Medical records	≥ 5	0.89 (0.40-1.98) ¹	Age, gender, race, year of admission	27
Mack <i>et al</i> ^[39] , 1986	1976-1981	Case-control	38/490	44/490	Hospital records	Pathology records	> 1	0.8 (0.5-1.4)	Age, gender, race	27
Cuzick and Babiker ^[40] 1989	1983-1986	Case-control	14/216	7/279	Hospital records	Medical records	NA	2.43 (0.91-7.12)	Age, gender	29
Farrow and Davis ^[41] 1990	1982-1986	Case-control	8/218	6/188	Hospital records	Cancer registry	≥ 3	1.1 (0.3-3.4)	Age	29
Bueno de Mesquite <i>et al</i> ^[42] , 1992	1984-1988	Case-control	24/176	44/487	Hospital records	Medical records	> 5	1.15 (0.55-2.40)	Age, response status, smoking	31
Lee <i>et al</i> ^[43] , 1996	1989-1994	Case-control	12/282	6/282	Hospital records	Medical records	NA	2.04 (0.76-6.21)	Age, gender	43
Gullo <i>et al</i> ^[45] , 1996	1987-1992	Case-Control	93/720	71/720	Hospital records	Medical records	> 1	1.00 (0.70-1.43)	Age, gender	34
Silverman <i>et al</i> ^[30] , 2001	1986-1989	Case-Control	132/484	150/2099	Hospital records	Pathology records	> 2	1.77 (1.26-2.48) ¹	Age, race, gender, smoking, alcohol consumption, BMI, Calorie intake.	31
Ko <i>et al</i> ^[32] , 2007	1995-1999	Case-control	75/532	155/1701	Hospital records	SEER abstracts	NA	1.73 (1.29-2.33) ¹	Ag, gender, BMI, smoking, diabetes	36
Hassan <i>et al</i> ^[44] , 2007	2000-2006	Case-Control	808	808	Hospital records	Self reported	> 2	OR = 1.1 (0.9-1.8)	Age, gender, smoking, comorbidities	35
Zhang <i>et al</i> ^[31] , 2014	1994-1998	Case-Control	215	676	Self report	Pathology reports	> 2	2.11 (1.32-3.35)	Age, gender, race, smoking, physical activity, diabetes	51
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	1106/33280	2572/100000	Medicare database	Cancer registry	> 6	OR = 1.23 (1.15-1.33)	Age, gender, diabetes	49
Ichimiya <i>et al</i> ^[26] , 1986	1953-1984	Cohort	3/1238	NA	National registry	Death registry	NA	SMR = 0.86 (0.33-2.25) ¹	Age, gender	33
Shibata <i>et al</i> ^[46] , 1994	1981-1990	Cohort	65/13979	NA	Hospital records	NA	> 4	RR = 2.09 (0.99-4.39)	Age, gender, smoking	32
Ekbom <i>et al</i> ^[33] , 1996	1965-1987	Cohort	261/62615	NA	National registry	Cancer registry	> 1	1.20 (1.06-1.36)	Age, gender	28
Chow <i>et al</i> ^[34] , 1999	1977-1993	Cohort	184/42461	NA	National registry	Cancer registry	≥ 4	1.3 (1.1-1.6)	Age, gender, obesity, years of follow-up, other comorbidities	33
Coughlin <i>et al</i> ^[35] , 2000	1982-1996	Cohort	3751/1.2 M	NA	Study database	Cancer registry	14	RR = 1.2 (1.0-1.5)	Age, gender, smoking, race, education, BMI, diet.	31
Ye <i>et al</i> ^[48] , 2001	1965-1997	Cohort	730/268312	NA	National database	Cancer registry	≥ 2	SIR = 1.06 (0.98-1.14)	Age, gender, calendar year	35
Schernhammer <i>et al</i> ^[47] , 2002	1976-1986	Cohort	37/145927	256/1675355	Self-report	Self report and death registry	> 10	1.23 (0.86-1.77)	Age, gender, BMI, Physical activity, diabetes	34
Goldacre <i>et al</i> ^[22] , 2005	1963-1999	Cohort	127/39254	791/334813	NHS database	Cancer registry	≥ 2	1.06 (0.88-1.26)	Age, gender, calendar year, residence.	36
Arnold <i>et al</i> ^[36] , 2009	1984-2004	Cohort	6243/1060389	NA	Hospital records	Death registry	NA	HR = 1.62 (1.02-2.55) black HR = 1.10 (1.0-1.22) white	Age, gender, BMI, smoking, FH of pancreatic cancer, diabetes.	41
Chen <i>et al</i> ^[29] , 2014	2000-2010	Cohort	16/5850		National database	Cancer registry	10	1.13 (0.60-2.12)	Age, gender, comorbidities	53

¹RR and 95% confidence intervals were calculated from raw data. NA: Not available; BMI: Body mass index.

into the duodenum can either reflux back into the stomach and oesophagus or proceed cephalad down

to the small and large bowel. Increased duodeno-gastro-oesophageal reflux after cholecystectomy is

Table 6 Descriptive characteristics of studies on the association between cholecystectomy and extrahepatic bile duct cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	118/3681	2572/100000	Medicare database	Cancer registry	> 6	OR = 1.19 (0.98-1.43)	Age, gender, diabetes	49
Chow <i>et al</i> ^[34] , 1999	1977-1993	Cohort	16/42461	NA	National registry	Cancer registry	≥ 4	0.7 (0.3-1.4)	Age, gender, obesity, years of follow-up, other comorbidities	33
Chen <i>et al</i> ^[29] , 2014	2000-2010	Cohort	9/5850		National database	Cancer registry	10	2.22 (0.91-5.41)	Age, gender, comorbidities	53

NA: Not available.

Table 7 Descriptive characteristics of studies on the association between cholecystectomy and liver cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	332/10219	2572/100000	Medicare database	Cancer registry	> 6	OR = 1.23 (1.15-1.33)	Age, gender, diabetes	49
Chow <i>et al</i> ^[34] , 1999	1977-1993	Cohort	48/42461	NA	National registry	Cancer registry	≥ 4	1.1 (0.7-1.5)	Age, gender, obesity, years of follow-up, other comorbidities	33
Goldacre <i>et al</i> ^[22] , 2005	1963-1999	Cohort	38/39245	306/334813	NHS database	Cancer registry	NA	0.91 (0.64-1.25)	Age, gender, calendar year, residence	36
Chen <i>et al</i> ^[29] , 2014	2000-2010	Cohort	87/5850	163/5850	National database	Cancer registry	10	1.17 (0.90-1.52)	Age, gender, comorbidities	53

NA: Not available.

Table 8 Descriptive characteristics of studies on the association between cholecystectomy and small intestinal cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	148/3694	2572/100000	Medicare database	Cancer registry	> 6	OR = 1.49 (1.26-1.77)	Age, gender	49
Lagergren <i>et al</i> ^[51] , 2001	1965-1997	Cohort	68/278460 ¹	NA	National registry	National registry	10	1.77 (1.37-2.24)	Age, gender, time aftercholecystectomy	38
Lagergren <i>et al</i> ^[51] , 2001	1965-1997	Cohort	98/278460 ²	NA	National registry	National registry	10	1.71 (1.39-2.08)	Age, gender, time aftercholecystectomy	38
Goldacre <i>et al</i> ^[52] , 2012	1998-2008	Cohort	NA	327460/3M	HES database	Cancer registry	10	2.47 (0.82-6.28)	Age, gender, period since cholecystectomy	45

¹Proximal small bowel adenocarcinoma; ²Distal small bowel carcinoids. NA: Not available.

controversial^[74-76] and probably relates to the method of measurement^[76]. The effects of refluxed bile may be augmented by additional noxious refluxed material such as acid and pancreatic enzymes^[77].

Bile acids were initially proposed as carcinogenic. However, later work with rodent models suggested that they should be regarded as cancer promoters (increasing tumorigenesis by other known carcinogens) rather than carcinogens acting independently^[78-80]. More recent evidence supports the view that bile acids (primary or secondary) are carcinogens in humans^[81,82]. Bile acids cause DNA damage probably indirectly through induction of oxidative stress and production of reactive oxygen species which damage DNA^[83]. Repeated DNA damage may increase the mutation rate including that of tumor suppressor genes

and oncogenes^[84]. Additional reports suggest that bile acids at an increased concentration induce apoptosis and hence select for apoptosis resistant cells^[85] with an increased rate of mutation^[86].

More than 95% of the bile salts synthesized in the liver are reabsorbed either by passive diffusion in the proximal jejunum, or by active transport in the distal ileum. The bile salts are then transported *via* the portal vein back to the liver where they are absorbed by hepatic cells and again secreted as bile. The enterohepatic recirculation of bile salts recycles about 6-8 times daily^[87]. The bile salts are the ionized form of the bile acid molecule. The carboxyl group in the side chain of the bile salt molecule when activated can react with glycine or taurine forming amides known as conjugated bile salts. Intestinal anaerobic

Table 9 Descriptive characteristics of studies on the association between cholecystectomy and colorectal cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	3907/150045	2572/100000	Medicare database	Cancer registry	> 6	OR = 0.97 (0.92-1.02)	Age, gender	49
Schmidt <i>et al</i> ^[60] , 2012	1992-1994	Case-Control	10/254	0/1043	National database	Cancer registry	24	HR = 1.20 (0.85-1.70)	Age, gender	41
Todoroki <i>et al</i> ^[67] , 1999	1991-1994	Case-Control	226/1982	270/2129	Medicare database and self report	Cancer registry	≥ 2	OR = 1.1 (0.9-1.3)	Age, gender, Family history, BMI, diet, NSAIDs	48
Zeng <i>et al</i> ^[62] , 1993	1965-1986	Case-Control	8/503	18/2188	Hospital records	Hospital records	≥ 2.5	OR = 1.95 (0.84-4.51)		26
Neugut <i>et al</i> ^[66] , 1991	1986-1988	Case-Control	11/106	41/507	Hospital Records	Self-report	2	OR = 0.96 (0.46-1.98)	Age, gender	34
Lee <i>et al</i> ^[54] , 1989	1980-1987	Case-Control	40/165	19/165	Hospital Records	Hospital Records	≥ 2	RR = 2.11 (1.19-3.85)		30
Kune <i>et al</i> ^[65] , 1988	1980-1981	Case-Control	35/715	57/727	Hospital Records	Self-reporting and hospital records		RR = 1.10 (0.7-1.1)		36
Neugut <i>et al</i> ^[58] , 1988	1983-1985	Case-Control	11/56	10/84	Hospital records	Self-reporting		OR = 1.8 (0.6-5.4)	Age, socioeconomic status	38
Friedman <i>et al</i> ^[63] , 1987	1971-1984	Case-Control	174/5898	773/27687	Medicare Database	Cancer registry	≥ 2	OR = 1.1 (0.9-1.2)	Age, gender, geographical area, calendar year	47
¹ Weiss <i>et al</i> ^[53] , 1982	1976-1977	Case-Control	92	687	Cancer Registry	Self-reporting	≥ 1	RR = 1.4 (0.7-2.6)		40
² Turnbull <i>et al</i> ^[61] , 1981	1972-1976	Case-Control	20/305	5	Hospital records	Hospital records	> 5	RR = 2.7		33
Chen <i>et al</i> ^[29] , 2014	2000-2010	Cohort	67/5850	76/5850	National database	Cancer registry	10	HR = 1.56 (1.12-2.17)	Age, gender, comorbidities	53
² Hartz <i>et al</i> ^[55] , 2012	1993-1998	Cohort	1207/150912	NA	National database	Self-report	8	HR = 1.36 (1.13-1.64)	Age, smoking, obesity, Family history, comorbidities	48
Shao <i>et al</i> ^[56] , 2005	1987-2002	Cohort	297/55960	574668	National database	National database		IRR = 1.32 (1.16-1.48)	Age, gender	54
² Schernhammer <i>et al</i> ^[59] , 2003	1982-1998	Cohort	133/6669	78515	National database of nurses	Self-report and National death registry	16	RR = 1.21 (1.01-1.46)	Age, smoking, BMI, lifestyle factors, comorbidities	57
¹ Johansen <i>et al</i> ^[64] , 1996	1977-1989	Cohort	225/42098	NA	Hospital database	Cancer registry	1-16	RR = 1.09 (1.0-1.2)	Age, gender, calendar year	43
Linos <i>et al</i> ^[57] , 1981	1950-1969	Cohort	42/1681		Hospital database	Hospital records and self reporting		² RR = 1.3 (0.9-1.9) ³ RR = 1.3 (0.7-2.2)		34

¹Excluding rectal cancer; ²Women only; ³Men only; NA: Not available; BMI: Body mass index; NSAIDs: Nonsteroidal anti-inflammatory drugs.

bacteria, for example species of the *Bacteroides fragilis* group, deconjugate and dehydroxylate the bile salts by removing glycine and taurine residues and the hydroxyl group at position 7^[14]. The primary bile salts are then biochemically transformed into the secondary bile acids, deoxycholic acid and lithocholic acid. The deconjugated and dehydroxylated bile salts are less soluble in intestinal chyme and are therefore less readily absorbed from the intestinal lumen than the bile salts that have not been subjected to bacterial metabolism. Based on both experimental and observational epidemiologic studies, deoxycholic acid has been classified as a potential tumor promoter in conjunction with other genotoxic agents^[88-90]. Studies of concentration levels of deoxycholic acid in both

fecal and serum samples have been associated with colorectal adenomas and cancer^[91-93]. The relatively prominent distribution of adenocarcinoma in the duodenum and proximal jejunum, particularly after cholecystectomy, has been attributed to proximity to the juncture of the common bile duct^[51].

The other culprits in this scenario include gut metabolic hormones. As an illustrative example, elevated circulating levels of Cholecystokinin (CCK) have been found after cholecystectomy^[94]. Normal human pancreas and pancreatic cancer have been found to possess receptors for CCK. CCK has been shown to stimulate the growth of human pancreatic cancer cell lines^[95] and initiate pancreatic carcinogenesis in rodents^[96].

Table 10 Descriptive characteristics of studies on the association between cholecystectomy and proximal colon cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	1963/66740	2,572/100000	Medicare database	Cancer registry	> 6	OR = 1.06 (0.99-1.12)	Age, gender	49
Todoroki <i>et al</i> ^[67] , 1999	1991-1994	Case-control	134/967	270/2129	Medicare database and self report	Cancer registry	≥ 2	OR = 1.3 (1.0-1.6)	Age, gender, family history, BMI, diet, NSAID use	48
Zeng <i>et al</i> ^[62] , 1993	1965-1986	Case-control	5/108	18/2188	Hospital records	Hospital records	≥ 2.5	OR = 5.85 (2.13-16.7)		26
Friedman <i>et al</i> ^[63] , 1987	1971-1984	Case-control	70/1925	773/27687	Medicare Database	Cancer registry	≥ 2	OR = 1.2 (0.9-1.5)	Age, gender, geographical area, calendar year	47
Vernick <i>et al</i> ^[69] , 1981	1975-1978	Case-control	21/150	23/250	National database	Self-report and hospital records		RR = 1.77 (0.95-3.3)		44
¹ Schernhammer <i>et al</i> ^[59] , 2003	1982-1998	Cohort	46/6669	78515	National database of nurses	Self-report and National death registry	16	RR = 1.35 (0.97-1.88)	Age, smoking, BMI, lifestyle factors, comorbidities	57
Lagergren <i>et al</i> ^[51] , 2001	1965-1997	Cohort	861/278460	NA	National registry	National registry	10	SIR = 1.16 (1.08-1.24)	Age, gender, time after cholecystectomy	35
¹ Ekbom <i>et al</i> ^[68] , 1993	1965-1983	Cohort	633/62615		National registry	National registry	< 23	SIR = 1.24 (1.03-1.48)	Age	46

¹Women only. NA: Not available; BMI: Body mass index; NSAIDs: Nonsteroidal anti-inflammatory drugs.

Table 11 Descriptive characteristics of studies on the association between cholecystectomy and distal colon cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Nogueira <i>et al</i> ^[21] , 2014	1992-2005	Case-control	986/40996	2572/100000	Medicare database	Cancer registry	> 6	OR = 0.93 (0.86-1.00)	Age, gender	49
Todoroki <i>et al</i> ^[67] , 1999	1991-1994	Case-control	87/965	270/2129	Medicare database and self report	Cancer registry	≥ 2	OR = 0.8 (0.6-1.1)	Age, gender, Family history, BMI, diet, NSAID use	48
Zeng <i>et al</i> ^[62] , 1993	1965-1986	Case-control	2/131	18/2188	Hospital records	Hospital records	≥ 2.5	OR = 1.87 (0.43-8.14)		26
Friedman <i>et al</i> ^[63] , 1987	1971-1984	Case-control	60/1963	773/27687	Medicare database	Cancer registry	≥ 2	OR = 1.2 (0.9-1.6)	Age, gender, geographical area, calendar year	47
Schernhammer <i>et al</i> ^[59] , 2003	1982-1998	Cohort	28/6669	78515	National database of nurses	Self-report and National death registry	16	RR = 0.95 (0.64-1.43)	Age, smoking, BMI, lifestyle factors, comorbidities	57
Lagergren <i>et al</i> ^[51] , 2001	1965-1997	Cohort	2564/278460	NA	National registry	National registry	10	SIR = 0.98 (0.94-1.02)	Age, gender, time after cholecystectomy	35

NA: Not available; BMI: Body mass index; NSAIDs: Nonsteroidal anti-inflammatory drugs.

DISCUSSION

This systematic review has found inconclusive evidence for an association between a history of cholecystectomy and cancers of the Gastro-intestinal tract at each site. The contradictory evidence was found both in case-control studies and in cohort studies. The same level of inconsistency was noted by meta-analyses in individual cancer sites. The most likely explanation for this level of inconsistency is the quality of studies. In general, case-control studies are more susceptible to selection bias than are cohort studies. This is mainly due to the increased surveillance of patients in cohort studies which is less likely to distort the true effect^[31]. Secondly, the majority of studies did not stipulate or report criteria for disease ascertainment. This was based mainly on

cancer or death registry data which are subject to errors. Thirdly, Adjustment for confounding factors has been variable amongst the studies but inadequate in the majority. It is very likely that the same risk factors for cholelithiasis and cancer such as obesity, diet, ethnicity, family history, cigarette smoking, education and physical activity co-exist. Unless such confounders are adjusted for it is difficult to conclude that the risk is purely a cholecystectomy effect.

It is established that early manifestations of abdominal cancers are sometimes misdiagnosed as gallstones and treated with cholecystectomy. Some studies have shown that a not uncommon cause of readmission after laparoscopic cholecystectomy is colon cancer^[18,97,98]. As such, all short term studies which did not adjust for the period between cholecystectomy and the incident cancer must be

Table 12 Descriptive characteristics of studies on the association between cholecystectomy and rectal cancer

Ref.	Period of study	Study design	No. of cases	No. of controls	Exposure ascertainment	Outcome ascertainment	Follow-up (yr)	Effect estimate	Adjustments	Quality of publication
Chiong <i>et al</i> ^[70] , 2012	1950-2012	Meta-Analysis	14226/460262	NA	Mixed sources	Mixed sources	Variable	OR = 1.14 (0.92-1.41)	Age, gender	54
Zeng <i>et al</i> ^[62] , 1993	1965-1986	Case-Control	1/264	18/2188	Hospital records	Hospital records	≥ 2.5	OR = 0.46 (0.06-3.45)		26
Kune <i>et al</i> ^[65] , 1988	1980-1981	Case-Control	29/715	57/727	Hospital records	Self-reporting and hospital records		RR = 1.22 (0.7-2.0)		36
Friedman <i>et al</i> ^[63] , 1987	1971-1984	Case-Control	43/1921	773/27687	Medicare database	Cancer registry	≥ 2	OR = 0.9 (0.6-1.2)	Age, gender, geographical area, calendar year	47
Weiss <i>et al</i> ^[53] , 1982	1976-1977	Case-Control	49	687	Cancer registry	Self-reporting	≥ 1	RR = 1.0 (0.4-2.4)	Age	40
Shao <i>et al</i> ^[56] , 2005	1987-2002	Cohort	83/55960	574668	National database	National database		IRR = 1.00 (0.85-1.17)	Age, gender	54
¹ Schernhammer <i>et al</i> ^[59] , 2003	1982-1998	Cohort	32/6669	78515	National database of nurses	Self-report and National death registry	16	RR = 1.58 (1.05-2.36)	Age, smoking, BMI, lifestyle factors, comorbidities	57
² Johansen <i>et al</i> ^[64] , 1996	1977-1989	Cohort	119/42098	NA	Hospital register	Cancer registry	1-16	RR = 1.07 (0.9-1.3)	Age, gender, calendar year	43
Linos <i>et al</i> ^[57] , 1981	1950-1969	Cohort	¹ 7/1681 ³ 4/1681		Hospital database	Hospital records and self reporting		¹ RR = 0.5 (0.1-1.3) ³ RR = 2.3 (0.9-4.8)		34

¹Women only; ²Excluding rectal cancer; ³Men only. NA: Not available; BMI: Body mass index.

viewed with caution. Further, if there is a causal relationship between cholecystectomy and cancer, the rate ratio, representing the rate in the cholecystectomy cohort relative to that in the comparison cohort, should increase over time (due to the latent period required for the development of a cancer) and the risk should remain at long time intervals. This has not been shown with any consistency in the reported studies.

Cholecystectomy is a common procedure throughout the world^[6-9]. The necessity for cholecystectomy has arisen mainly due to symptomatic gallstone disease which is age related^[2]. Equally, gastro-intestinal cancers are common and increase with increasing age^[81,99]. The association between cancers of the gastro-intestinal tract is more likely to be a casual rather than a causal. In order to establish a causal association, the criteria of Sackett's modification of the Bradford-Hill criteria would need to be applied on epidemiological research^[100]. There are to-date no Randomised controlled trials which have arisen to confirm nature of the association nor is it feasible to conduct such trials in the short term. The strength of the association appears weak at best, particularly when taking into account the almost universal lack of adjustment for all necessary confounders. There is lack of consistency of the association in several cohort studies with some showing an association in a positive direction and others confirming the null hypothesis of an association. Although all the studies show a temporal relationship between cholecystectomy and cancer, there is an equal temporal relationship with

the gallstones phenotype. In terms of the plausibility of the association, a number of studies have proposed mechanisms for carcinogenesis by either bile salts or enteric hormones. These studies are based on *in-vitro* or animal experiments and have concluded that bile salts are either promoters increasing tumorigenesis by other known carcinogens^[79,80] or carcinogens acting independently^[82]. A possible objection to the contention that bile acids could be carcinogenic is based on evolutionary grounds. For a natural substance produced by the body, to be carcinogenic is counter intuitive. Hence the emphasis on bile acids being promoters of other known carcinogens or acting in high physiologic concentrations in certain individuals after high fat intake^[101]. With regards to enteric hormones, the evidence was based mainly on *in-vitro* experiments. In terms of biological plausibility, it seems contrary to our understanding of how natural selection operates, that a natural substance produced by the body for a beneficial purpose could be carcinogenic. On the basis that none of the criteria have been to-date satisfactorily satisfied that no such causal relationship exists between cholecystectomy and gastro-intestinal tract cancers. It seems more likely that some of the gallstone producing phenotype, develop gastro-intestinal tract cancers as they age.

This review has several potential limitations. Although an extensive search was made of all the available literature, it is possible that some articles were accidentally missed. However, having captured the majority if not all of the available articles on the

subject, it seems less likely that any missed articles would alter the conclusions made. Although it is difficult to rule out publication bias, there appears to be a reasonable number of epidemiological studies from different parts of the world, which encompass the cholecystectomy cohort with no significant differences between populations. Thirdly, a number of the publications reported in this review are of moderate quality but a reasonable number are of sufficiently higher quality. In addition, the majority of reported studies suffer from heterogeneity.

This review has included a number of historical articles on the subject. In a subject with so few articles on each of the components of the GIT, it was important to include such historic articles to avoid bias acknowledging that the inclusion of such articles would not alter the conclusion. It is reasonable to conclude that if a real effect were apparent, it would have manifested more strongly.

In conclusion, this systematic review has found contradictory evidence of an association between a history of cholecystectomy and gastro-intestinal tract cancers. Based on current evidence, there is no clear association between cholecystectomy and cancers of the gastro-intestinal tract. Additional robust, scientific studies are warranted.

COMMENTS

Background

Cholecystectomy for gallstone disease is a common operation. A number of studies have investigated the association between cholecystectomy and/or cholelithiasis with gastro-intestinal tract cancers with contradictory results.

Research frontiers

To the best of our knowledge, no such comprehensive systematic review of the association between cholecystectomy and gastro-intestinal tract (GIT) cancers has previously been published. The objective of this study was to review systematically all the studies which have investigated the association between cholecystectomy and GIT cancers.

Innovations and breakthroughs

A number of systematic reviews have been published which were focused on one or other type of GIT cancers, this is the first comprehensive systematic review which have addressed all GIT cancers and have added comments on mechanisms of carcinogenesis in different parts of the GIT.

Applications

Based on the lack of clear association between cholecystectomy and GIT cancers, clinicians can be assured of the benefits of cholecystectomy without the risk of GIT cancer. In consenting patients for cholecystectomy, clinicians can assure patients that no causal risk of GIT cancers after cholecystectomy was demonstrated.

Terminology

Carcinogenesis is the formation of cancer driven either by direct carcinogens which act independently to cause mutations or by promoters which drive cellular proliferation without causing mutations themselves. As such promoters require the field to have been exposed to a tumor initiator which could be mutagenic.

Peer-review

This is a comprehensive review of the world's literature highlighting the relationship between prior cholecystectomy and gastro-intestinal malignancies by site as well as proposed mechanism/pathogenesis.

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Association between cadherin-17 expression and pathological characteristics of gastric cancer: A meta-analysis

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Abstract

AIM: To construct a meta-analysis in order to examine the relationship between cadherin-17 (CDH17) and

gastric cancer (GC).

METHODS: Related articles were selected by searching the following English or Chinese electronic databases: CINAHL, MEDLINE, Science Citation Index, the Chinese Journal Full-Text, and the Weipu Journal. Newcastle-Ottawa Scale (NOS) criteria were used to ensure consistency in reviewing and reporting results. Statistical analyses were conducted with Version 12.0 STATA statistical software.

RESULTS: Ultimately, 11 articles, with a total of 2,120 GC patients, were found to be eligible for study inclusion. In comparisons of GC patients by TNM stage (III-IV vs I-II: OR = 2.35, 95%CI: 1.15-4.825, $P = 0.019$), histologic grade (3-4 vs 1-2: OR = 3.48, 95%CI: 1.36-8.92, $P = 0.009$), invasion grade (T3-4 vs T1-2: OR = 2.86; 95%CI: 1.69-4.83; $P = 0.000$), and lymph node metastasis (positive vs negative: OR = 2.64; 95%CI: 1.33-5.27; $P = 0.006$), it was found that CDH17 showed more positive expressions in each of the more severe cases. Country-stratified analyses from all four experimental subgroups showed that high CDH17 expression levels may be related to GC among Chinese and Korean populations (all $P < 0.05$), with the exception of the invasion grade T3-4 vs T1-2 comparison, where the relation only held among the Chinese population (OR = 2.86, 95%CI: 1.69-4.83, $P = 0.000$).

CONCLUSION: Collectively, the data reflects the capacity of CDH17 in tumor proliferation and metastasis among GC patients.

Key words: Cadherin 17; Protein expression; Gastric cancer; Meta-analysis

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Core tip: This meta-analysis conducted in order to examine the relationship between cadherin-17 (CDH17) and gastric cancer (GC), with the data reflecting the capacity of CDH17 in tumor proliferation and metastasis in GC patients.

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INTRODUCTION

Gastric cancer (GC) is a malignant disease arising from gastric tissue. More than 90% of GCs are adenocarcinomas, with the remainder being lymphomas or emerging from gastrointestinal stromal tissue (sarcomas)^[1]. GC is the fourth most common cancer in the world and the second leading cause of cancer-related death globally^[2]. Although the last decade has witnessed a dramatic decrease in the prevalence of GC in some developed countries, approximately one million newly diagnosed cases, along with 800000 GC-related deaths, occur each year worldwide^[3]. In fact, it has been reported that two-thirds of GC cases occur in developing countries, with particularly high incidences in areas such as Japan, China, Central America, and South America^[4]. Generally, gastric carcinogenesis is a multifactorial process involving the participation of both environmental and epigenetic factors^[1,5]. A variety of factors that are related to the occurrence and progression of GC have been confirmed, including *Helicobacter pylori* infection, low fruit intake, consumption of foods high in salt, smoking, and consumption of preserved foods and nitrites^[4,6,7]. Recently, adhesion molecules such as cadherin-17 (CDH17), which is reported to be implicated in tumor invasion and metastasis, have been shown to be correlated with GC pathogenesis^[8].

CDH17, also known as liver-intestine cadherin or human peptide transporter-1, consists of seven homologous repeated domains, and while it belongs to the cadherin superfamily responsible for intercellular conjunction, its structure is distinct from that of classic cadherin family members^[9]. CDH17 possesses the ability to modulate Ca²⁺-dependent homophilic cell-cell adhesion without depending on cytoskeleton interaction, suggesting that it may play a central role in tumor metastasis^[10,11]. In addition, CDH17 has been shown to both act as a peptide transporter and participate in the development of the embryonic gastrointestinal tract^[12]. In recent studies, CDH17 expression was observed to be upregulated in GC patients, implying that CDH17 expression is related to

GC development^[5,9]. In general, CDH17 is expressed on the basolateral surface of enterocytes and goblet cells in the small and large intestines in a selective manner, and is seldom detected in the healthy adult stomach or liver^[10,13]. Cadherins, including CDH17, are single-pass transmembrane proteins that function mainly in cell-cell adhesion and may be implicated in tumorigenesis^[14]. It has been reported that CDH17 knockdown may result in the inactivation of Wnt signaling, which could in turn inhibit the activity of cancer cell invasion^[12]. More importantly, there is evidence indicating that loss of CDH17 may lead to an increased expression of placental growth factor and metal-responsive transcription factor-1, which is believed to increase tumor aggression, thus modulating angiogenesis in human carcinoma^[15]. Additionally, by activating the NFκB signaling pathway, CDH17 can also induce lymph node (LN) metastasis, as well as the formation of tumors in GC^[16]. With this in mind, it is possible that CDH17 expression may be involved in the pathogenesis and progression of GC. Several clinical studies have documented that high expression levels of CDH17 were positively connected with histological stage, tumor invasion, and LN metastasis of GC, revealing that CDH17 expression might be a valuable indicator for predicting the progression and prognosis of GC^[8,17]. However, other researchers have failed to find evidence to support the correlation of CDH17 expression with pathological characteristics of GC^[5,9]. With such an inconsistency in previous reported findings in mind, we performed the current meta-analysis with the available data in order to clarify the connection between CDH17 expression and the pathological features of GC.

MATERIALS AND METHODS

Literature search

The following computerized bibliographic databases were reviewed, without restrictions with respect to language or data collection, to identify relevant articles relating to the association of CDH17 expression and GC susceptibility: PubMed, Embase, CINAHL, Science Citation Index, the Cochrane Library, Current Contents Index, Chinese Biomedical, the Chinese Journal Full-Text, and the Weipu Journal. The search terms "stomach neoplasms" or "gastric cancer" or "stomach cancer" or "gastric neoplasms" or "gastric carcinomas" or "stomach carcinomas" or "carcinoma ventriculi" or "stomach neoplasms" and "CDH17 protein, human" or "CDH17" or "liver-intestine-cadherin" or "cadherin-17" were entered in the databases searches as medical subject heading terms and text words, within a highly sensitive search strategy. Manual searches were used to screen other eligible studies.

Study selection

After reading the abstract, full texts were retrieved and assessed for their suitability based on the following

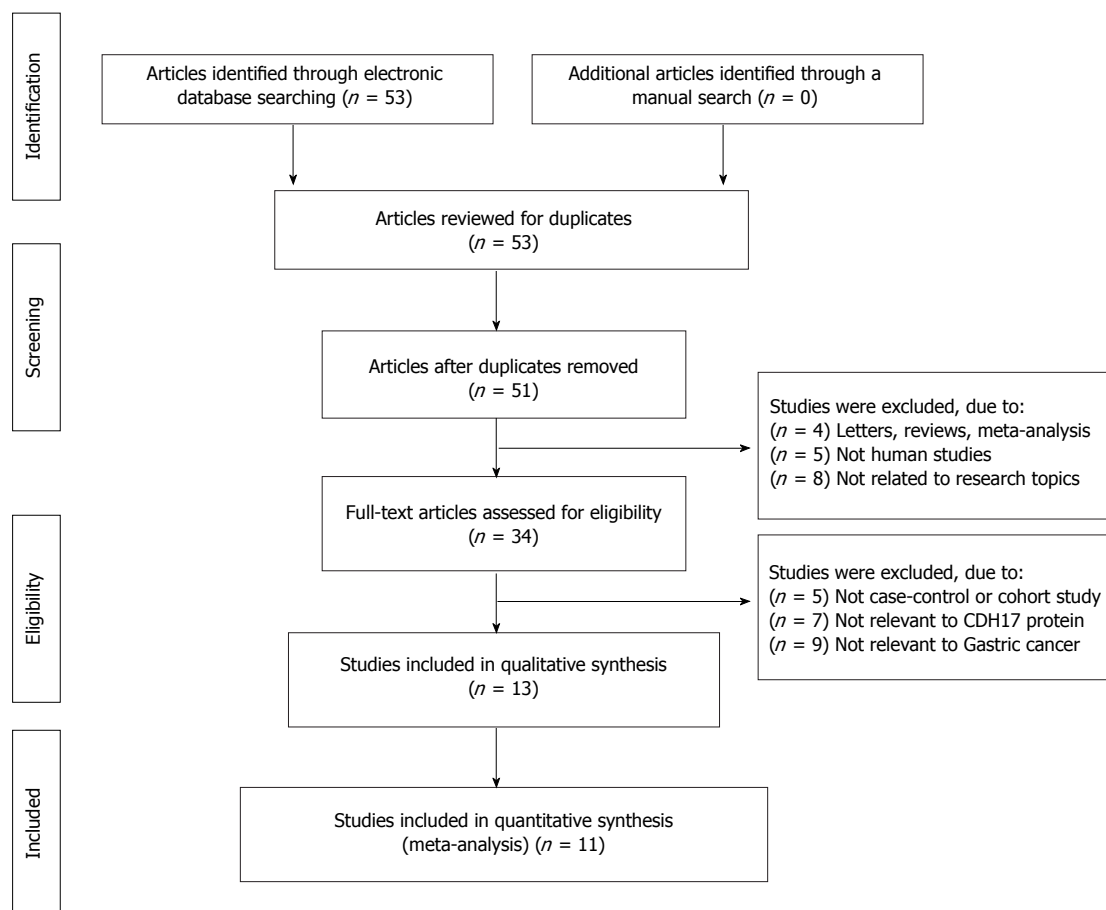


Figure 1 Flow chart showing study selection procedure. Eleven final case-controlled studies were included in this meta-analysis.

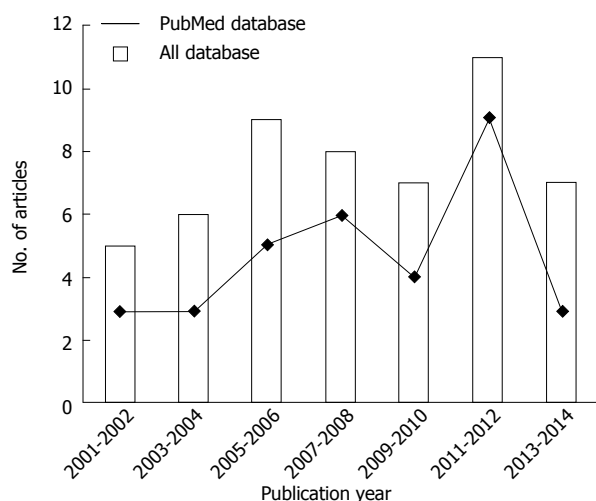


Figure 2 Distribution of topic-related literature in the electronic database over the last decade.

inclusion criteria: (1) clinical studies had to examine the association between CDH17 expression and the pathological features of GC within a human population; (2) tumor specimens were drawn from all patients confirmed to have GC *via* histological examination, and the pathological staging for each GC sample was classified in accordance with the TNM system^[18]; (3) the article must be published in a peer-reviewed journal

and provide original data; and (4) the article must supply sufficient information on CDH17 expression. The major exclusion criteria were: (1) article did not satisfy the inclusion criteria designed in the current study; (2) studies turned out to be abstracts, reviews, case report, letters, meta-analyses, or proceedings; (3) duplication publications or studies with overlapping data; and (4) subgroup analysis of the included trials.

Data extraction and quality assessment

A standard reporting form was used to extract data from each included study, with the collected descriptive information including: first author surname and initials, year of submission, country, racial descent, study design, number of cases and controls, demographic variables, CDH17 detection method, CDH17 expression, and confirmation of diagnosis. Two reviewers independently assessed the methodological quality of the included trials using the Newcastle-Ottawa Scale (NOS) criteria to ensure consistency in reviewing and reporting results^[19]. Three aspects were considered in the NOS criteria: (1) subject selection: 0-4; (2) subject comparability: 0-2; and (3) clinical outcome: 0-3. NOS scores range from 0 to 9; a score ≥ 7 indicated that the study was of good quality. Disagreement on the inclusion of a single study was settled either by discussion or after consultation with a third investigator.

Table 1 Characteristics of included studies focusing on protein expression of cadherin-17

Ref.	Year	Country	Sample	Gender (M/F)	Age (yr)	Sample	Method	NOS score
Lin <i>et al</i> ^[17]	2014	China	216	150/66	65 (32-84)	Tissue	EnVision	8
Qiu <i>et al</i> ^[9]	2013	China	156	103/53	57 (27-78)	Tissue	EnVision	7
Wang <i>et al</i> ^[16]	2012	China	191	117/74	-	Tissue	Non-EnVision	8
Sakamoto <i>et al</i> ^[5]	2012	Japan	152	-	-	Tissue	Non-EnVision	7
Wang <i>et al</i> ^[30]	2011	China	264	157/107	-	Tissue	EnVision	8
Liu <i>et al</i> ^[29]	2011	China	46	37/9	-	Tissue	EnVision	6
Lee <i>et al</i> ^[28]	2010	United States	440	-	-	Tissue	Non-EnVision	8
Xu <i>et al</i> ^[27]	2009	China	215	169/46	57 (24-82)	Tissue	Non-EnVision	8
Ge <i>et al</i> ^[35]	2008	China	166	109/57	52.2 ± 10.2	Tissue	EnVision	7
Tian <i>et al</i> ^[25]	2007	China	66	32/34	53 (29-91)	Tissue	Non-EnVision	6
Park <i>et al</i> ^[8]	2007	South Korea	208	135/73	-	Tissue	Non-EnVision	8

NOS: Newcastle-Ottawa Scale.

Statistical analysis

The association between CDH17 expression and the pathological features of GC was estimated by the odds ratio (OR) and a 95% confidence interval. We used Cochran's *Q*-statistic ($P < 0.05$ was considered significant) and I^2 tests to quantify heterogeneity^[20]. In order to calculate the pooled ORs, fixed/random effects models were used; a random effects model was applied in the event of significant heterogeneity ($P < 0.05$ or I^2 test exhibited $> 50\%$), while ORs were pooled *via* the fixed-effects model^[21,22]. In the event of significant heterogeneity, subgroup analysis was performed to find potential explanatory variables for the differences. In addition, we employed sensitivity analyses to evaluate whether a single study had the weight to impact the overall estimate. The effect of publication bias was determined *via* Egger's linear regression test ($P < 0.05$ was considered significant), which can be used to evaluate funnel plot asymmetry; an asymmetric plot reveals possible publication bias^[23,24]. Statistical analyses were conducted with STATA statistical software (Version 12.0, Stata Corporation, College Station, TX, United States).

RESULTS

Selection of eligible studies

Figure 1 shows the flow chart of identified publications and the main reason for exclusion. Initially, 53 potential articles emerged from the electronic databases. Of the 53 articles, 2 studies were duplicates and thus removed. After title/abstract screening, 17 irrelevant studies were also removed. A further 21 studies were excluded after detailed readings during full text assessment, thereby leaving 13 remaining studies for qualitative analysis. Based on this analysis, 2 additional studies were removed. This left 11 case-controlled studies published between 2007 and 2014 for the meta-analysis^[5,8,9,17,25-31] (Figure 2).

Demographic variables

The 13 include studies consisted of 2,120 total GC patients with sample sizes ranging from 46 to 440.

Sample sizes > 200 were considered "large". Of the 11 included studies, only one operated within a Caucasian population [United States (Lee HJ)]; the remaining 10 studies were conducted in Asian populations [China (Lin Z, Qiu HB, Wang J, Wang B, Liu SQ, Xu XY, Ge J, and Tian MM), Japan (Sakamoto N), and Korea (Park SS)]. Two studies (Lee HJ and Sakamoto N) did not provide gender information, while six studies (Wang J, Sakamoto N, Wang B, Liu SQ, Lee HJ, and Park SS) failed to obtain age information. There was only one non-Asian study, though it had a large sample size. The detection of CDH17 expression was divided into EnVision and non-EnVision groups (PV, LSAB, SP, and ABC). CDH17 expression in different pathological stages (TNM stage, histologic grade, invasive grade, and LN metastasis) and baseline characteristics for the 11 individual studies are summarized in Table 1.

Meta-analysis of the MMP-3 levels with GC risk

In the meta-analysis, the relationship between CDH17 expression and the pathological features of GC was assessed *via* the random effect model for observed heterogeneity (TNM III-IV vs I-II: $I^2 = 88.4\%$, $P = 0.000$; histologic grade 3-4 vs 1-2: $I^2 = 91.1\%$, $P = 0.000$; invasive grade: T3-4 vs T1-2: $I^2 = 59.7\%$, $P = 0.030$; LN metastasis: $I^2 = 85.2\%$, $P = 0.000$). Results showed that CDH17 exhibited more positive expression in patients with TNM III-IV staging GC than in those with III-IV staging GC based on the ORs from the combined results of all the included studies (OR = 2.35; 95%CI: 1.15-4.82; $P = 0.019$). We also examined the role of CDH17 expression in the histologic grade of GC progression, and found that GC tissues from histologic grades 3-4 had higher CDH17 expression than those with histologic grades 1-2 (OR = 3.48; 95%CI: 1.36-8.92; $P = 0.009$). As for the invasive grade, we found that CDH17 expression was significantly higher in GC tumors with an invasion depth of T3-4 when compared with the T1-2 (OR = 2.86; 95%CI: 1.69-4.83; $P = 0.000$). Meanwhile, GC tissues with LN metastases had higher CDH17 expression than those with no detectable LN metastases (OR = 2.64; 95%CI: 1.33-5.27; $P = 0.006$) (Figure 3).

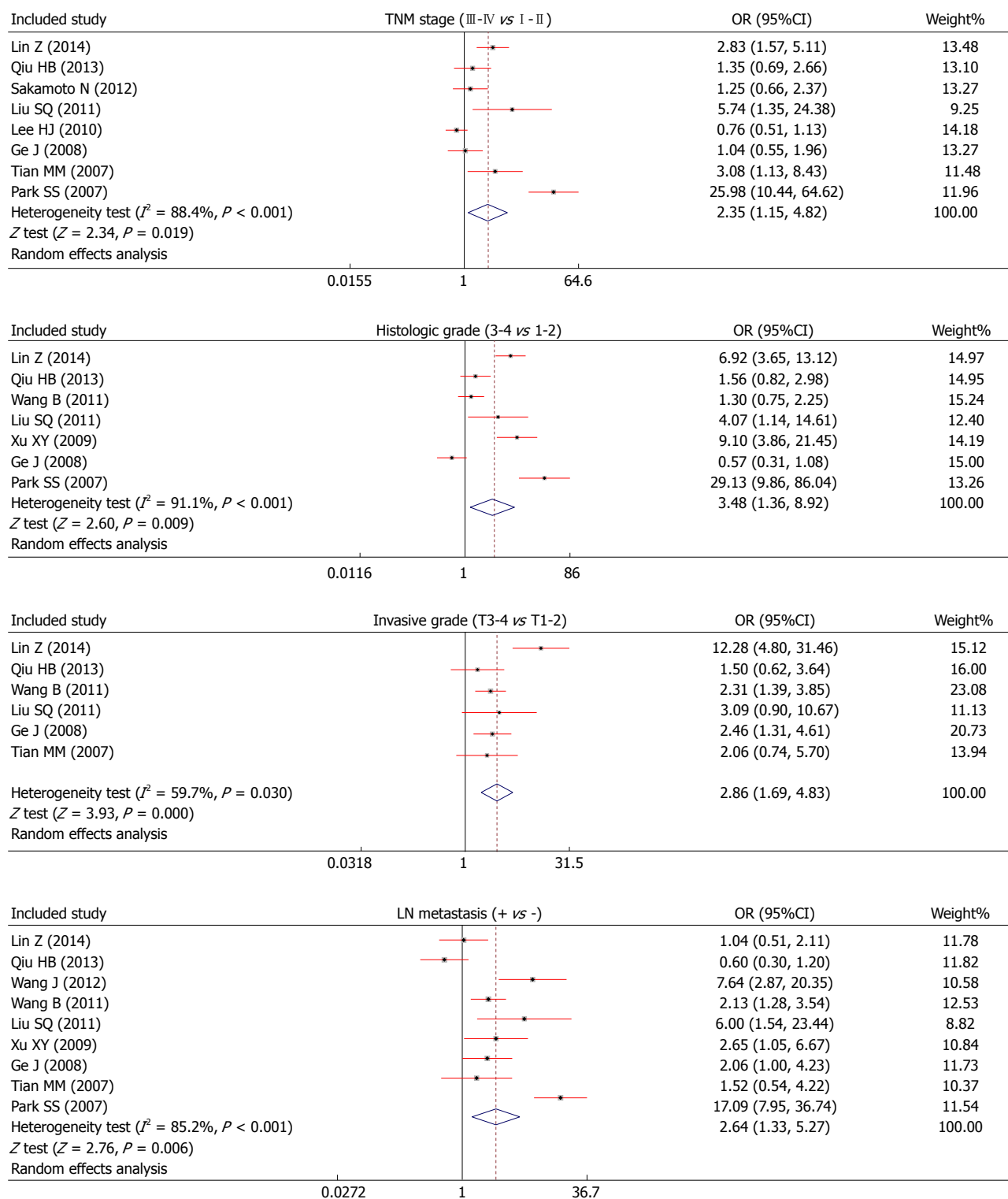
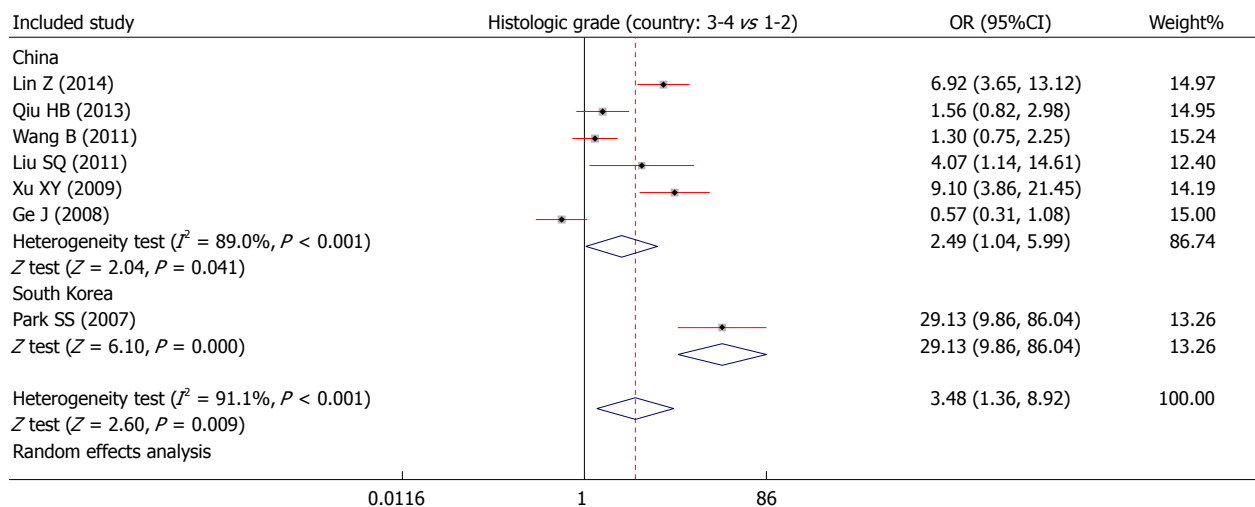
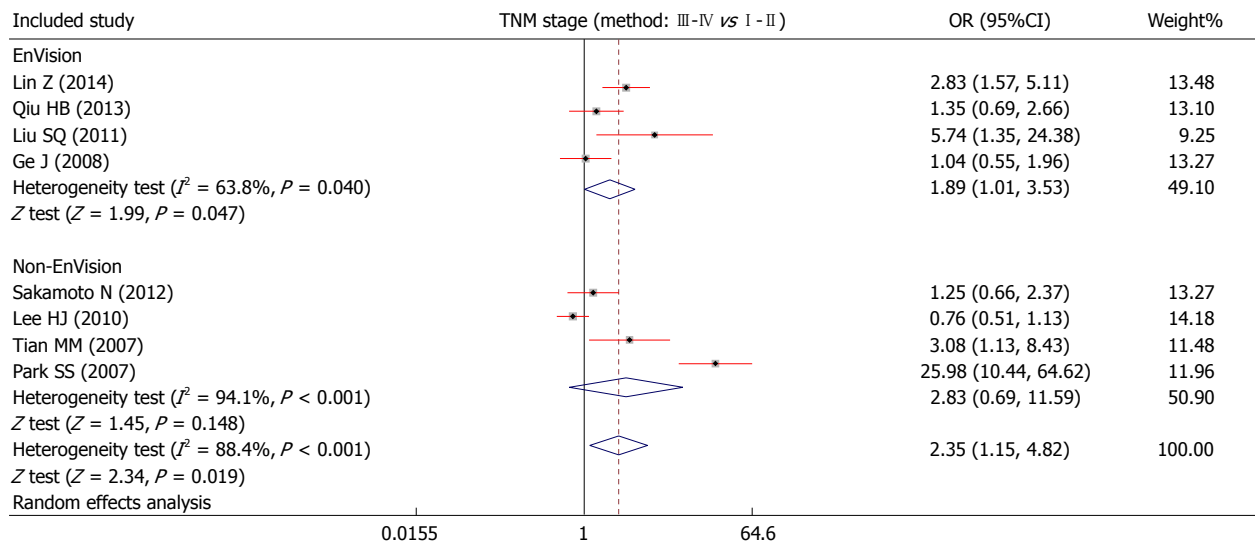
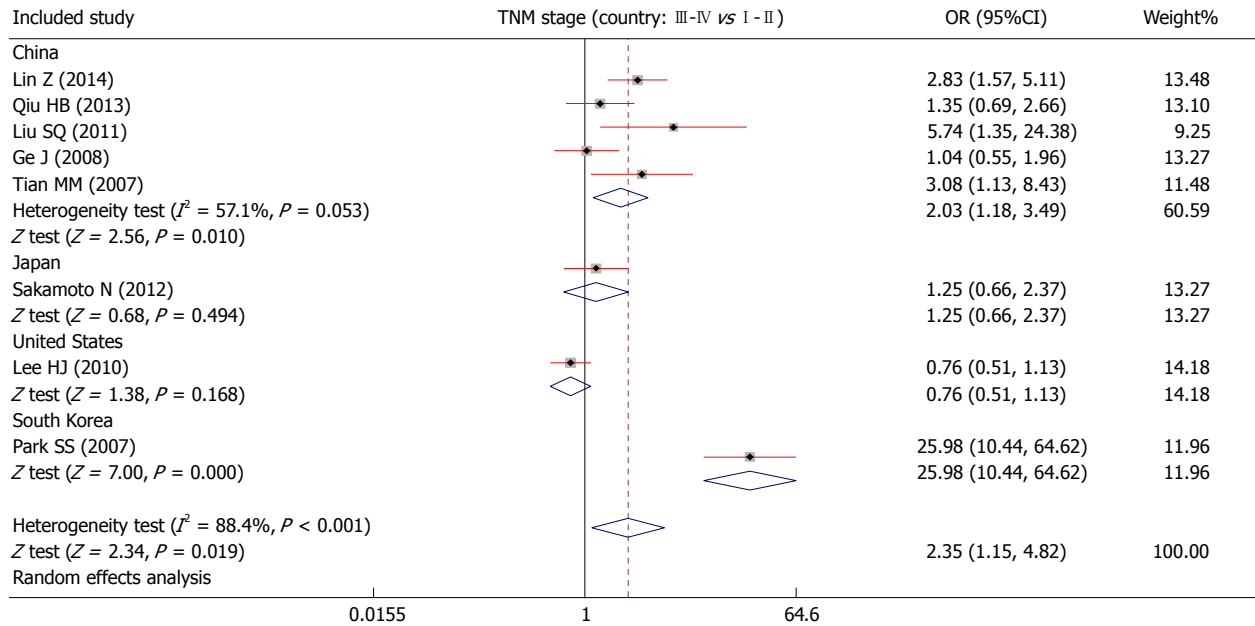


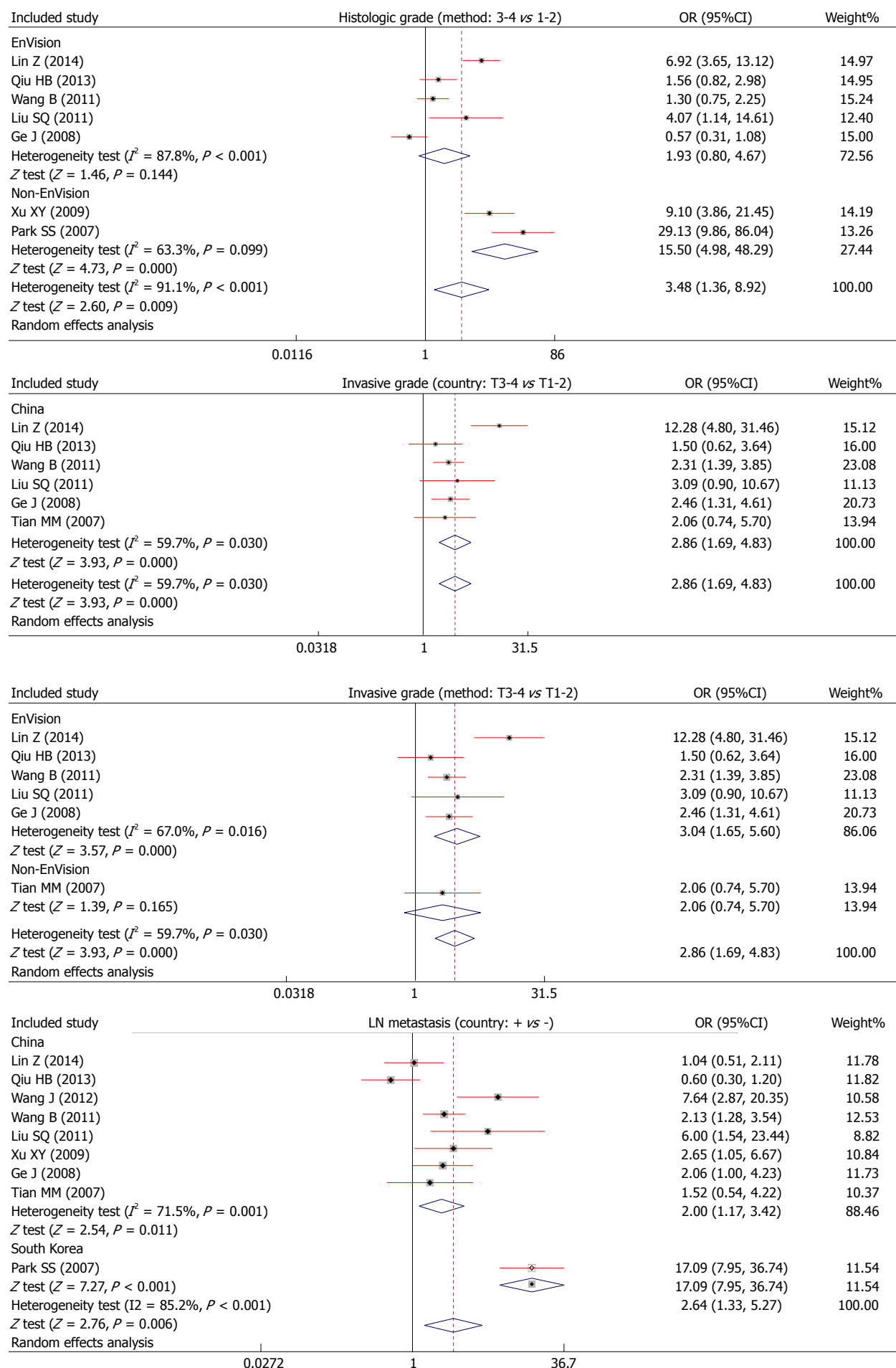
Figure 3 Forest plots for the relationships between cadherin-17 protein expression and the pathological characteristics of gastric cancer.

Subgroup analysis

Since heterogeneity was found, the relationship between CDH17 expression and the pathological features of GC were evaluated for subgroups of different explanatory variables. We found that CDH17 expression occurred more frequently in TNM III-IV staging GC than those with I-II staging GC in the China (OR = 2.03; 95%CI: 1.18-3.49; $P = 0.010$) and Korea subgroups (OR = 25.98; 95%CI: 10.44-64.62;

$P = 0.000$), but not in the Japan (OR = 1.25; 95%CI: 0.66-2.37; $P = 0.494$) or United States subgroups (OR = 0.76; 95%CI: 0.51-1.13; $P = 0.168$). In addition, we found positive associations between CDH17 expression and histologic grade of GC in the China (OR = 2.49; 95%CI: 1.04-5.99; $P = 0.041$) and Korea subgroups (OR = 29.13; 95%CI: 9.86-86.04; $P = 0.000$). Significant differences in CDH17 expression were also observed between samples with T3-4 grade





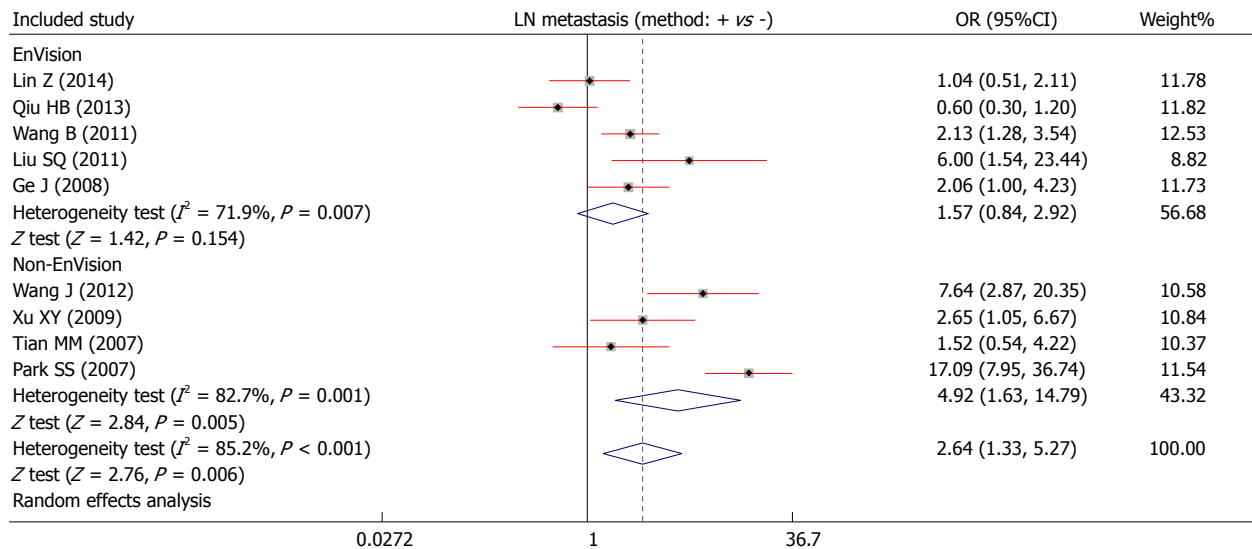


Figure 4 Subgroup analyses of the relationship between cadherin-17 protein expression and the pathological characteristics of gastric cancer.

GC and T1-2 grade GC in the China subgroup (OR = 2.86; 95%CI: 1.69-4.83; $P = 0.000$). In addition, the results showed significant difference in CDH17 expression between LN metastasis-positive samples and LN metastasis-negative GC samples in both the China (OR = 2.00; 95%CI: 1.17-3.42; $P = 0.011$) and Korea subgroups (OR = 17.09; 95%CI: 7.95-36.74; $P = 0.000$).

In the method-classified subgroup analysis, we revealed significant differences in CDH17 expression in GC patients with TNM stage III-IV when compared with those with TNM stage I-II in the EnVision subgroup (OR = 1.89; 95%CI: 1.01-3.53; $P = 0.047$), but not in the non-EnVision subgroup (OR = 2.83; 95%CI: 0.69-11.59; $P = 0.148$). Meanwhile, evidence suggested that GC patients with overexpressed CDH17 were associated with a higher histologic grade in the non-EnVision subgroup (OR = 15.50; 95%CI: 4.98-48.29; $P = 0.000$), but not in the EnVision subgroup (OR = 1.93; 95%CI: 0.80-4.67; $P = 0.144$). Additionally, the results also indicated that CDH17 expression occurred more frequently in GC patients with a higher invasive grade in the EnVision subgroup (OR = 3.04; 95%CI: 1.65-5.60; $P = 0.000$), but a similar association was not found in the non-EnVision subgroup (OR = 2.06; 95%CI: 0.74-5.70; $P = 0.165$). Furthermore, we observed increased CDH17 expression in GC patients with LN-positive metastasis relative to those with LN-negative metastasis in the non-EnVision subgroup (OR = 4.92; 95%CI: 1.63-14.79; $P = 0.005$), but not in the EnVision subgroup (OR = 1.57; 95%CI: 0.84-2.92; $P = 0.154$) (Figure 4).

Sensitivity analysis and publication bias

Sensitivity analyses were performed, with the results showing that no single study had the weight to impact the overall estimate of the association between

CDH17 expression and the pathological features of GC (Figure 5). We did not observe any obvious asymmetry from the shapes of the funnel plots, and the Egger's regression test suggested the absence of publication bias, with the exception of the association between CDH17 expression and TNM stage ($t = 2.49$; $P = 0.047$); thus, no significant publication bias was detected in the association of CDH17 expression with histologic grade ($t = 1.72$; $P = 0.146$), invasive grade ($t = 0.58$; $P = 0.594$), or LN metastasis ($t = 0.83$; $P = 0.435$) in our systematic reviews (Figure 6).

DISCUSSION

In this meta-analysis, the relationship between high CDH17 expression and pathological features of GC was observed. From the results, we can conclude there is a significant connection between CDH17 and TNM stages, histologic grade, invasive grade, and LN metastasis of GC. Single-pass transmembrane cadherins are a type of cell adhesion molecule that can regulate adhesion to adjacent cells depending on Ca^{2+} , thereby contributing to homophilic cell adhesion and tumor development^[32]. CDH17, a member of the cadherin superfamily that is expressed exclusively on enterocyte basolateral surfaces and intestine goblet cells (but not liver or stomach cells), differs from classic cadherins in structure and function^[33]. Different from classic cadherins such as E-, P-, and N-cadherins, CDH17 consists of seven cadherin type repeats without the His-Ala-Val motif of the N-terminal domain, has only 20 amino acid residues for its CDH17 cytoplasmic portion, has a short COOH-terminal for possible cell adhesion function, and displays no homology to classical cadherins which have the highly conserved 150-160 amino acid residues^[8]. The independent adhesion function from the cytoskeletal anchorage of CDH17 has no connection with catenins, actins, or

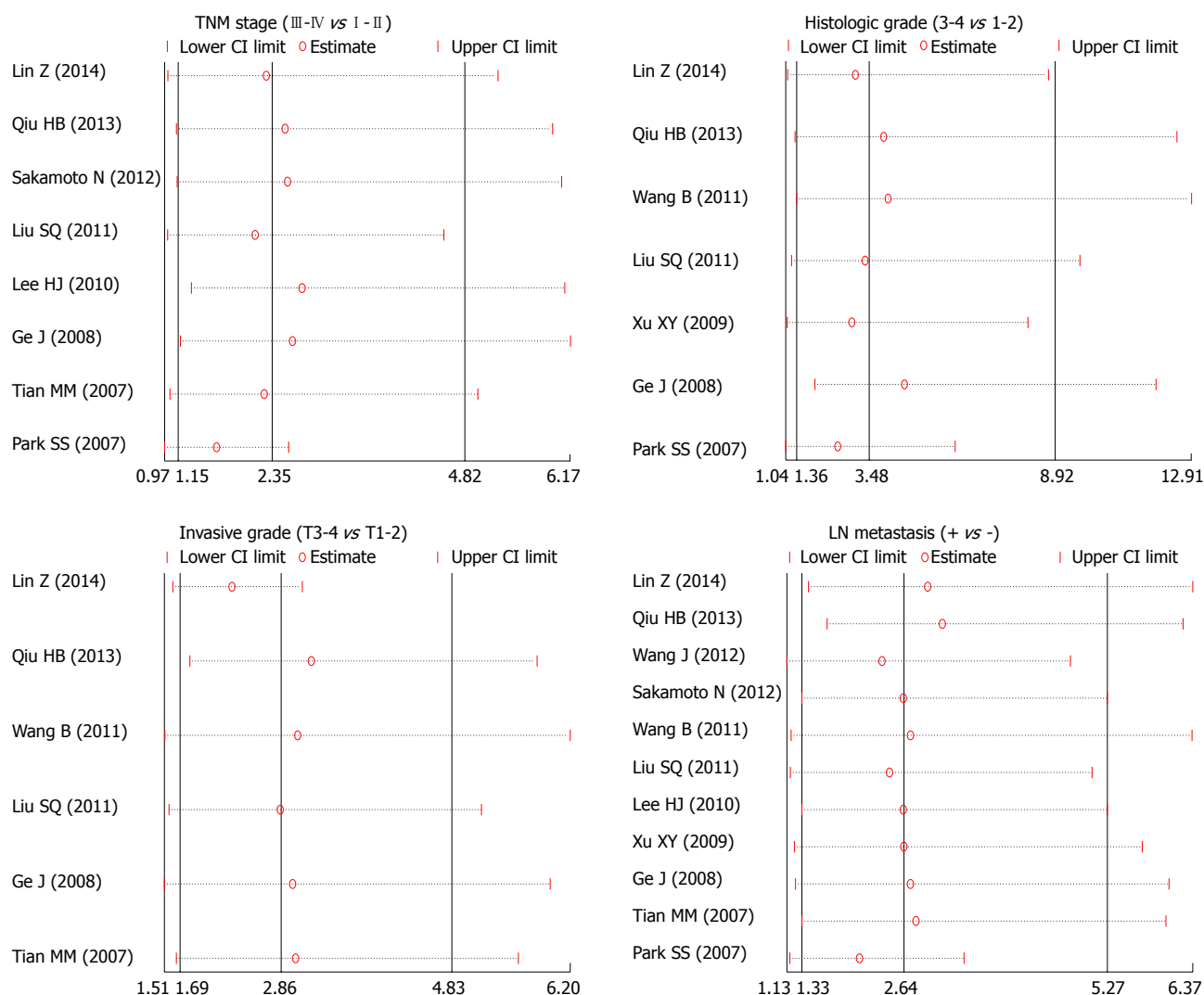


Figure 5 Sensitivity analysis of the summary odds ratio coefficients on the association between cadherin-17 protein expression and the pathological characteristics of gastric cancer.

other cytoplasmic components; this is vastly different from classical cadherins, suggesting a complementary classical cadherin adhesion function^[34]. It has been reported that the high expression of CDH17, as opposed to the low levels of other classical cadherins, is related to gastric cancer, colorectal cancer, liver cancer, pancreatic cancer, and cholangiocarcinoma, suggesting that CDH17 might play an important part in tumor progression^[33]. A series of studies have shown that the high expression of CDH17 is linked with the stage of the tumor, histological grade, invasive depth of the tumor, and lymph node metastasis of GC^[5,31]. The reason for high CDH17 expression levels in this capacity may be related to the activation of the NF κ B signaling pathway, which impacts processes such as p50/p65 heterodimer regulation of the transcription of responsive genes and differentiation of lymphatic endothelium into VEGFs. Relatedly, increased nuclear translocation of p65 in GC has a close relationship with tumor invasion depth and tumor metastases^[16]. Another mechanism may involve the Wnt/ β -catenin

pathway. It was found that the knockdown of CDH17 had the ability to decrease phosphorylation of GSK-3 β and β -catenin related with the reduction transactivation activity of TCF/LEF, thus decreasing the expression of cyclin-D1, which is of great importance in promoting cell proliferation and inhibiting cell apoptosis^[9,35]. Furthermore, CDH17 over-expression can activate the Ras/Raf/MEK/ERK MAPK signaling pathway, which may regulate cell proliferation, apoptosis, metabolism, and differentiation, which are all processes important in tumor biology^[17]. From the above analysis, we may draw the conclusion that the high expression of CDH17 is largely related to the pathological features of GC through three signaling pathways in the cells: the NF κ B signaling pathway, the Wnt/ β -catenin pathway, and the Ras/Raf/MEK/ERK MAPK signaling pathway. In agreement with our conclusion, Ge *et al.*^[26] also found that the expression of CDH17 may play an important part in the development of GC, suggesting a suitable marker for the prognosis of GC.

Given the fact that several factors may affect the

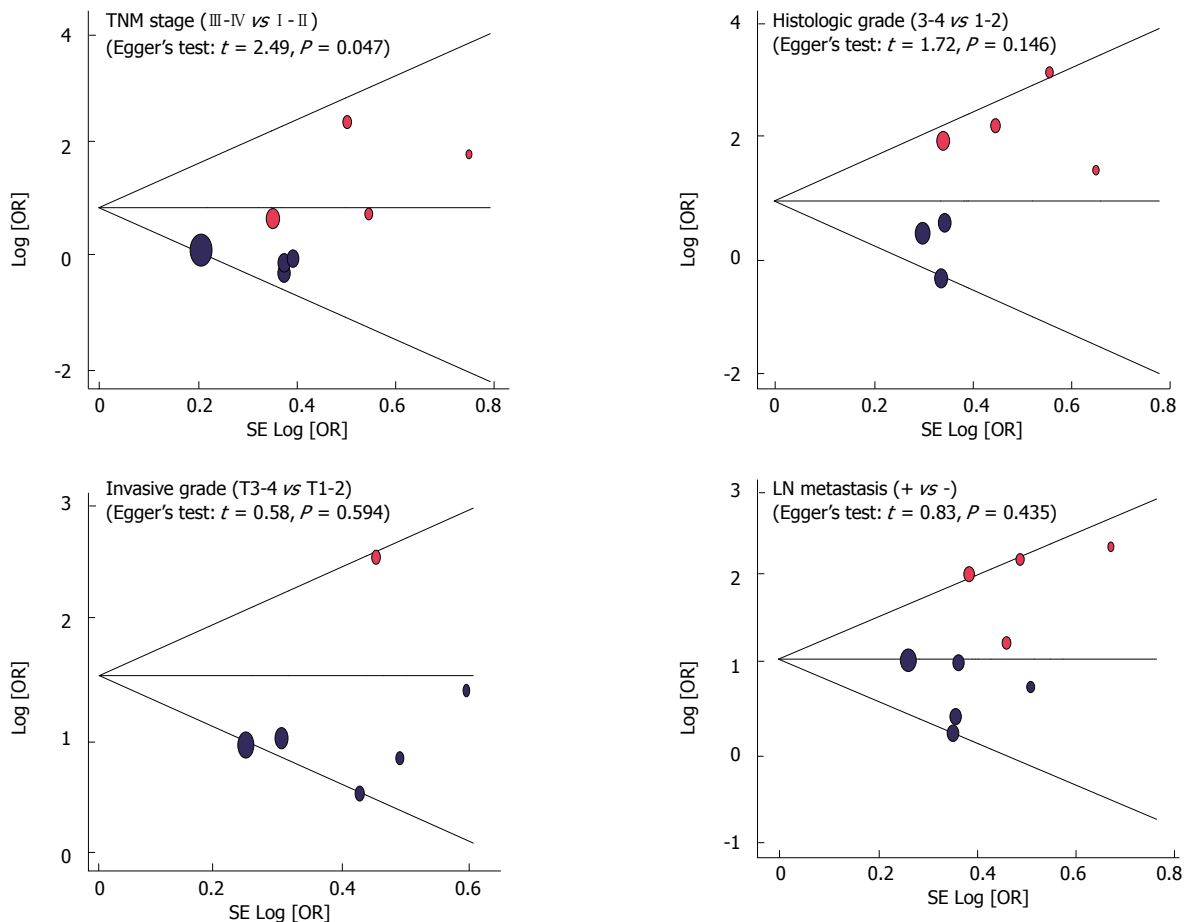


Figure 6 Funnel plot of publication biases on the association between cadherin-17 protein expression and the pathological characteristics of gastric cancer.

linkage between CDH17 expression and pathological features of GC patients, a stratified analysis based on country and detection method was conducted. From the country-stratified analysis, we found no obvious influence in either Chinese or Korean populations in TNM stages, histologic grade, invasive grade, or LN metastasis of GC. In Japan and the United States, however, the relationship was not as obvious. This can be explained by the differences in the environmental backgrounds and gene pools of these different populations. In conclusion, our results are partly in accordance with previous studies that hold that high expression of CDH17 has a typical connection with the pathological features of GC patients.

Our study does have some limitations that merit additional investigation. Firstly, our study is observational research that is cross-sectionally designed. Secondly, the sample sizes in more than half of the total involved articles were relatively small, and the number of patients in some groups was low. In particular, the small patient numbers in the stratified analyses of country and method may detract from the study results. Thirdly, there was a difference in the high/low cut-off values of CDH17 expression levels in the various included studies. The different cut-off values of

CDH17 between studies may influence the final results and be responsible for a few inconclusive outcomes. Finally, the existence of heterogeneity may also harm the integrity of results. A large number of the included studies were designed with small sample sizes, six studies lacked complete gender and age information, and the CDH17 expression detection methods were different from each other (EnVision, PV, LSAB, SP, and ABC). In this regard, differences in age, sex, and detection methods may be largely responsible for heterogeneity. Additionally, there was only one non-Asian study, and although it had a large sample size, this might have influenced the scope of our results. Considering the limitations listed above, the results, as well as the analysis, must be interpreted with caution.

In conclusion, the present study identified that CDH17 is an actual oncogene that plays an important role in cell proliferation, tumor growth, invasion, and metastasis in GC. This data may present a novel therapeutic approach in treating GC by targeting CDH17. Our conclusions, however, need to be confirmed due to the aforementioned limitations, *via* a combination of more and larger sample size publications, which use a consistent definition for cut-off values.

COMMENTS

Background

Cadherin-17 (CDH17) was detected to be overexpressed in gastric cancer (GC) and related to tumor incidence and recurrence, invasion and metastasis, advanced tumor stage, and poor survival in GC patients. To date, the cellular function and signaling mechanisms of CDH17 in GC remain inconsistent.

Research frontiers

The cellular function and signaling mechanisms of CDH17 in GC remain inconsistent.

Innovations and breakthroughs

The present study identified that CDH17 is an actual oncogene that plays an important role in cell proliferation, tumor growth, invasion, and metastasis in GC.

Applications

These data may present a novel therapeutic approach against GC by targeting CDH17.

Terminology

Odds ratio (OR) and a 95% confidence interval (95%CI) were used to evaluate specified relationships. The Cochran's Q-statistic and I^2 test were used to evaluate potential heterogeneity among studies.

Peer-review

The review is well written and deals with an important topic. This study found that the cadherin 17 protein is associated with the growth and metastasis of gastric cancer, suggesting a potential of targeted therapy against the protein. The analysis is interesting and important.

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Prognostic value of c-Met in colorectal cancer: A meta-analysis

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Abstract

AIM: To assess the prognostic value of c-Met status in colorectal cancer.

METHODS: We conducted a search in PubMed, Web of Science, and the Cochrane Library covering all published papers up to July 2014. Only studies assessing survival in colorectal cancer by c-Met status were included. This meta-analysis was performed by using STATA11.0.

RESULTS: Ultimately, 11 studies were included in this analysis. Meta-analysis of the hazard ratios (HR)

indicated that patients with high c-Met expression have a significantly poorer overall survival (OR) (HR = 1.33, 95%CI: 1.06-1.59) and progression-free survival (PFS) (HR = 1.47, 95%CI: 1.03-1.91). Subgroup analysis showed a significant association between high c-Met expression and poorer overall survival in the hazard ratio reported (HR = 1.41, 95%CI: 1.08-1.74).

CONCLUSION: The present meta-analysis indicated that high c-Met expression was associated with poor prognosis in patients with colorectal cancer.

Key words: Colorectal cancer; Prognosis; c-Met; Meta-analysis; Overall survival

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Core tip: High c-Met expression was found in colorectal cancer and showed a positive relationship with early tumor invasion and metastasis. However, there still seems to be no consensus about the prognostic properties of c-Met status. In this paper, after combining the data from 11 retrospective studies with 1,895 patients, the authors found that high c-Met expression was associated with poor prognosis in patients with colorectal cancer.

Liu Y, Yu XF, Zou J, Luo ZH. Prognostic value of c-Met in colorectal cancer: A meta-analysis. *World J Gastroenterol* 2015; 21(12): 3706-3710 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3706.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3706>

INTRODUCTION

Colorectal cancer (CRC) is one of the most common types of cancer worldwide. The 5-year survival rate of CRC is higher in the early stage due to radical surgical

resection. However, many patients who underwent resection for primary colorectal cancer developed local recurrences or distant metastases, and had a shorter survival^[1]. Recent treatment options for patients with advanced colorectal cancer included combining anti-epidermal growth factor receptor (EGFR) or anti-vascular endothelial growth factor monoclonal antibodies with chemotherapy. Although many predictive markers have been identified^[2-4], most of them are not commonly used in clinical practice. Identification of factors that can predict a more accurate prognosis is therefore required.

C-Met is a receptor tyrosine kinase encoded by the c-Met oncogene. High expression of c-Met has been found in different solid tumors and has a correlation with poor prognosis. In CRC, c-Met is considered to be related to tumor aggressiveness and invasiveness, as well as metastatic potential and poor prognosis^[5-7]. In many tumors, the c-Met signaling pathway is activated aberrantly and represents one of the most important mechanisms of progression and invasiveness^[8].

However, despite a large number of studies having researched the relationship between c-Met and survival in colorectal cancer, there still seems to be no consensus about the prognostic properties of c-Met status. It is for this reason that we performed this systematic review.

MATERIALS AND METHODS

Publication search

We searched PubMed, Web of Science, and the Cochrane Library with the following terms: (c-Met AND (colon or rectal or colorectal) AND (carcinoma or tumor or cancer) AND prognosis) from 1990 to July 2014. To expand our search, references of the retrieved articles were also screened for additional studies. The inclusion criteria for primary studies were as follows: (1) proven diagnosis of CRC in humans; (2) overall survival (OS) or progression-free survival (PFS) analyzed by c-Met level; and (3) c-Met evaluation using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) or immunohistochemistry (IHC). Data from abstracts, review articles, and letters were excluded.

Data abstraction

Relevant data were extracted from eligible studies by two researchers independently. The following information was extracted from each study: (1) basic information such as first author's name, country, and publication year of article; and (2) variables such as number of patients analyzed, disease stage, methods of c-Met analysis, and hazard ratio (HR) with 95%CI for progression-free survival (PFS) and overall survival (OS). When HRs and confidence intervals were not reported directly, we estimated them from the number of patients in each group and Kaplan-Meier curves by using the published methodology^[9].

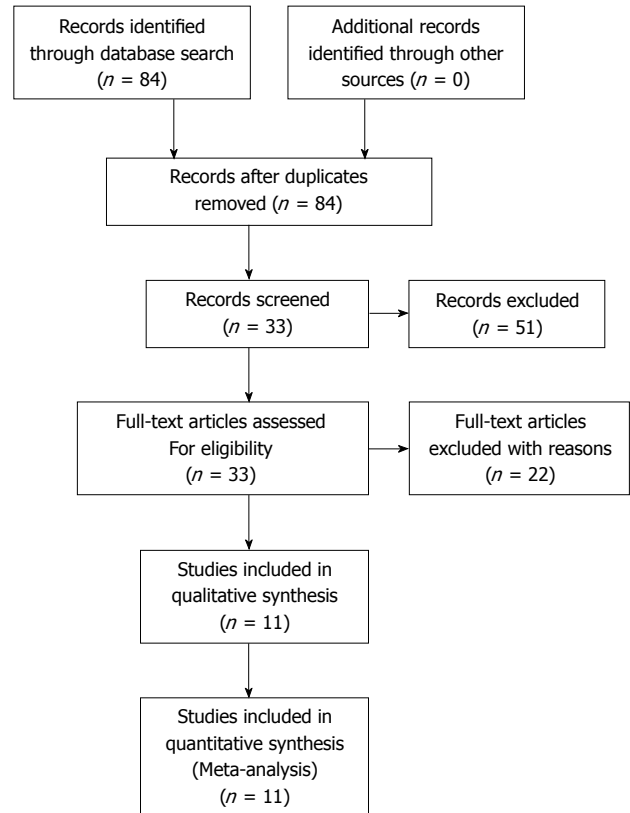


Figure 1 Flow chart of the meta-analysis.

Statistical analysis

We calculated HRs with their 95%CI to evaluate the relationships between c-Met level and PFS or OS. Heterogeneity was defined as $I^2 > 50\%$. When heterogeneity was judged among primary studies, the random-effects model was used. Otherwise, the fixed-effects model was used. Publication bias was assessed using Egger's test. All statistical analysis was carried out with STATA 11.0.

RESULTS

Characteristics of included studies

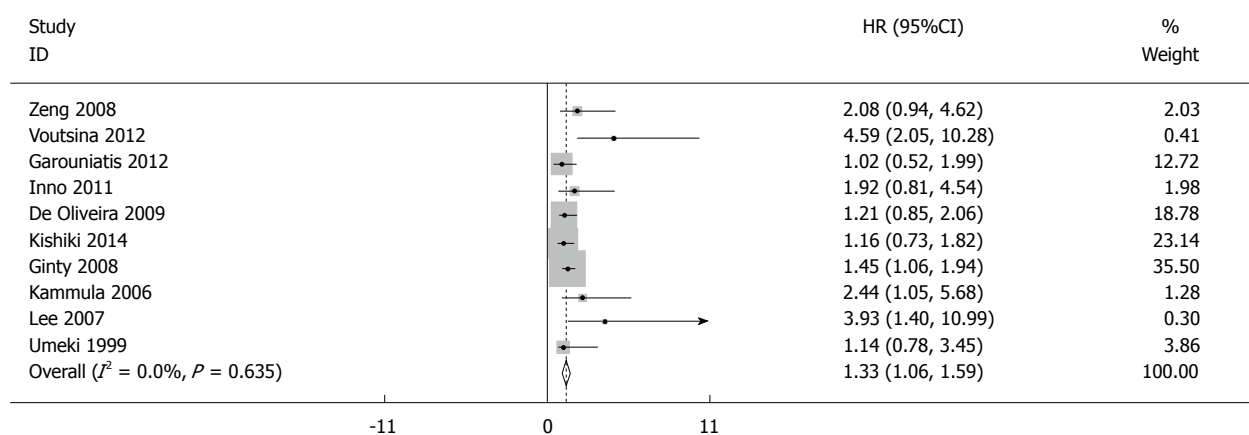
The initial search yielded 84 articles, of which 51 were excluded after screening of their titles and abstracts. Following the evaluation of the full text, a total of 11 studies were ultimately included in our study. The selection process for the studies involved in this meta-analysis is shown in Figure 1. From the 11 studies that were included^[10-20], a total of 1,895 patients were analyzed. The characteristics of the involved studies are showed in Table 1.

All of these studies were retrospective research. Of the 11 studies, two^[11,13] included only patients with advanced disease (stage IV), while the remaining 9 studies^[10,12,14-20] included patients with stage I-IV. None of the patients received therapy before resection of the primary tumor. C-Met evaluation was performed by IHC or PCR. The rate of high c-Met level ranged

Table 1 Characteristics of the selected studies

Ref.	No. of patients	Country	Method to stratify c-Met status	Stage	Stage (I, II)	High c-Met expression	Survival	HR	95%CI
Zeng <i>et al</i> ^[17] , 2008	247	United States	RT-qPCR	I-IV	46%	29%	OS	2.08 ¹	0.94-4.62
Voutsina <i>et al</i> ^[111] , 2012	73	Greece	IHC	IV	NS	52%	OS	4.59	2.05-10.28
Resnick <i>et al</i> ^[19] , 2004	134	Israel	IHC	I-IV	NS	77%	PFS	0.81 ¹	0.24-3.75
Garouniati <i>et al</i> ^[12] , 2012	183	Greece	IHC	I-IV	61%	72%	OS	1.02 ¹	0.52-1.99
Inno <i>et al</i> ^[13] , 2011	73	Italy	IHC	IV	NS	75%	PFS/OS	2.17/1.92	0.99-4.76/0.81-4.54
De Oliveira <i>et al</i> ^[14] , 2009	286	Brazil	IHC	I-IV	53%	79%	PFS/OS	1.65 ¹ /1.21 ¹	0.56-4.21/0.85-2.06
Kishiki <i>et al</i> ^[10] , 2014	75	Japan	IHC	I-IV	NS	48%	PFS/OS	1.46/1.16	1.06-2.02/0.73-1.82
Ginty <i>et al</i> ^[6] , 2008	583	United States	IHC	I-IV	46%	62%	OS	1.45	1.06-1.96
Kammula <i>et al</i> ^[18] , 2006	63	United States	RT-qPCR	I-IV	39%	81%	OS	2.44	1.05-5.68
Lee <i>et al</i> ^[15] , 2007	135	Taiwan	IHC	I-IV	NS	72%	PFS/OS	10.05/3.93	2.54-43.84/1.40-10.99
Umeki <i>et al</i> ^[20] , 1999	43	Japan	IHC/RT-qPCR	I-IV	35%	30%/12%	OS	1.14 ¹	0.78-3.45

¹HR estimated, if the HRs were not directly given in the studies, we calculated them from the survival curves. RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction; IHC: Immunohistochemistry; NS: Not shown.

**Figure 2** Fixed-effects model of hazard ratio of overall survival associated with c-Met overexpression.

from 12% to 81% (median, 61%). The median follow-up time was 80 months (range from 25 to 140 mo). In most of these studies, c-Met expression level was evaluated by cell percentage. High c-Met expression was defined as >50% positive cells. OS was presented in 10 studies, and PFS was reported in five studies. Six studies^[10,12,13,15,16,18] presented data on HR, with 95% CI for PFS or OS directly. The remaining five studies did not present HRs and 95% CIs directly, so we estimated them from Kaplan-Meier curves.

Association between c-Met and OS

Forest plots for the relationships between c-Met level and overall survival are showed in Figure 2, with the results indicating a significant relationship between high c-Met expression and poorer OS (overall HR = 1.33, 95%CI: 1.06-1.59). No significant heterogeneity was found ($P = 0.635$, $I^2 = 0.0\%$) and the HR for OS was assessed by using the fixed-effects model. There was no significant publication bias in this analysis of OS (Begg's test, $P = 0.052$; Egger's test, $P = 0.094$).

We also performed subgroup analysis in studies by HR reported. We found significant association between high c-Met expression and poorer overall survival

in the hazard ratio reported (HR = 1.41, 95%CI: 1.08-1.74).

Association between c-Met and PFS

The pooled HR for PFS showed that patients with a high c-Met level had a significantly poorer PFS (HR = 1.47; 95%CI: 1.03-1.91). No significant heterogeneity was found ($P = 0.778$, $I^2 = 0.0\%$), and the pooled HR for PFS was assessed by using the fixed-effects model (Figures 3 and 4). There was no significant publication bias in this analysis of PFS (Begg's test, $P = 0.462$; Egger's test, $P = 0.548$).

DISCUSSION

The main cause of CRC-related death is metastases. Identification of patients who are at risk of developing distant metastases is important to cancer treatment and prognosis. c-Met overexpression or genetic alteration has been proven to play an important role in the pathogenesis of many tumor types. In CRC, overexpression of c-Met has been found to be associated with tumor progression.

This systematic review is based on 11 studies

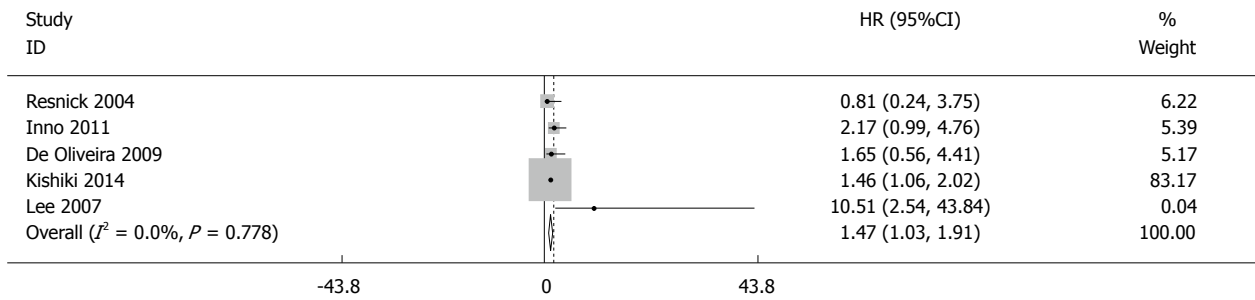


Figure 3 Fixed-effects model of hazard ratio of progression-free survival associated with c-Met overexpression.

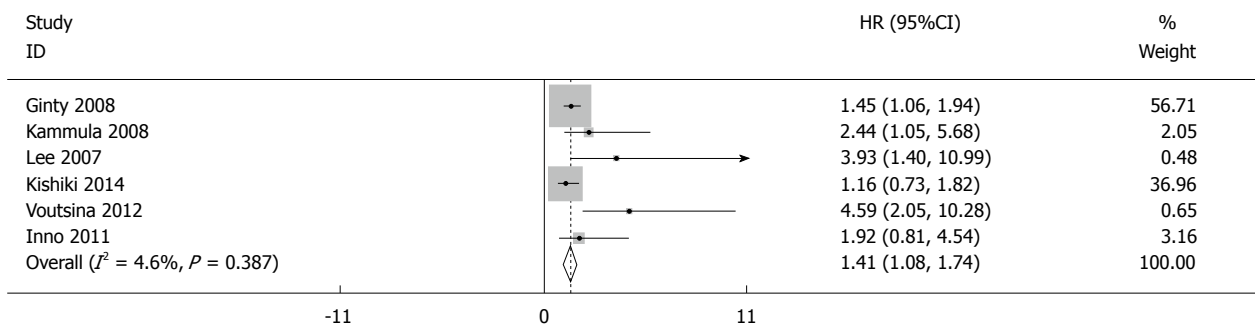


Figure 4 Forest plot showing the meta-analysis of hazard ratio reported for overall survival in patients.

and includes 1,895 patients with CRC. The results of this meta-analysis showed the prognostic value of c-Met expression level in CRC patients. High c-Met expression significantly predicted poor OS and PFS. We also performed subgroup analysis in studies by HR reported. There was a significant relationship between high c-Met expression and poorer overall survival in the hazard ratio reported.

A study conducted by Kishiki *et al.*^[10] showed that c-Met overexpression was associated with shorter PFS in metastasis colorectal cancer (mCRC) patients with wild-type *KRAS*. All patients received cetuximab- or panitumumab-based therapy. However, the study was conducted retrospectively in a relatively small and heterogeneous population. 90% of the patients were treated with two or more chemotherapy regimens before they were given anti-EGFR treatment. In addition, the anti-EGFR treatment protocols were also heterogeneous. Therefore their findings needed to be validated by more prospective studies. Zeng *et al.*^[17] found that amplification of c-Met gene is a relatively rare event (3.6%) in CRC, and that the majority of amplified cases occurred in patients with synchronous hepatic metastases (stage IV).

After identification of c-Met involvement in cancer progression and metastasis, many *in vitro* experimental studies have showed that c-Met plays a role in resistance to anti-EGFR therapy. Inno *et al.*^[13] retrospectively evaluated a cohort of 73 patients with mCRC treated with a cetuximab-containing regimen. They found an association of high c-Met level with shorter PFS and OS in patients with mCRC, and that the c-Met pathway may be involved in primary

resistance to cetuximab. However, their study cannot ascertain whether c-Met is a predictive biomarker, because they assessed only patients treated with a cetuximab-containing regimen. In CRC, many studies have proved that *KRAS* mutations predict unresponsiveness to EGFR-targeted monoclonal antibody therapies; however, there still about 26% of patients who are not responsive to EGFR-targeted therapy that are a wild-type for *KRAS*. Therefore, we hypothesize resistance to EGFR-targeted therapies may be mediated by the activation of parallel pathways such as the c-Met signaling pathway. Therefore more prospective studies are needed to affirm the results.

c-Met as a biomarker might be used to select advanced colorectal cancer patients who could benefit from targeted therapies. A growing number of studies from *in vitro*, *in vivo*, and in various stages of clinical testing have shown that c-Met tyrosine kinase (TK) inhibitors can block c-Met signaling and arrest or reverse tumor growth in a subset of human cancers^[21,22]. Recently a few studies have shown that c-Met amplification confers high sensitivity to a specific c-Met TK inhibitor in lung cancer and gastric cancer^[23,24]. All of these findings indicate that amplified c-Met may serve as a biomarker for targeted therapy.

Some limitations to this meta-analysis require particular note: heterogeneity; differences in clinical treatment; and different criteria to stratify c-Met status.

In conclusion, our meta-analysis demonstrated a significant association between high c-Met expression and poor OS and PFS in CRC for the first time. However, further larger prospective studies are needed

to confirm these results.

COMMENTS

Background

High c-Met expression was found in colorectal cancer and showed a positive relationship with early tumor invasion and metastasis.

Research frontiers

There still seems to be no consensus about the prognostic properties of c-Met status.

Innovations and breakthroughs

This is the first known paper to conduct a comprehensive meta-analysis with the aim of investigating the relationship between c-Met and prognosis of colorectal cancer. The authors found that high c-Met expression was associated with poor prognosis in patients with colorectal cancer.

Applications

This study furthers the understanding of the association of c-Met with colorectal cancer prognosis.

Peer-review

This is a well-performed meta-analysis that aims to assess the prognostic value of c-Met status in colorectal cancer. Its findings are interesting.

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Association of cholesterol with risk of pancreatic cancer: A meta-analysis

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Author contributions: Wang J designed the study, collected the data, performed the statistical analysis and wrote the manuscript as the first author; Wang WJ and Zhai L contributed to discussion and wrote the manuscript; and Zhang DF designed the study, contributed to discussion and edited the manuscript as the corresponding author.

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literature database for relevant articles published in English or Chinese. Pooled relative risks (RRs) with 95% confidence intervals (CIs) were calculated with a random-effects model.

RESULTS: We included 14 published articles with 439355 participants for dietary cholesterol, and 6 published articles with 1805697 participants for serum TC. For the highest vs lowest category of dietary cholesterol, the pooled RR (95%CI) of pancreatic cancer was 1.308 (1.097-1.559). After excluding two studies (RR > 3.0), the pooled RR (95%CI) was 1.204 (1.050-1.380). In subgroup analysis stratified by study design, the pooled RRs (95%CIs) were 1.523 (1.226-1.893) for case-control studies and 1.023 (0.871-1.200) for cohort studies. The association of dietary cholesterol with the risk of pancreatic cancer was significant for studies conducted in North America [1.275 (1.058-1.537)] and others [2.495 (1.565-3.977)], but not in Europe [1.149 (0.863-1.531)]. No significant association [1.003 (0.859-1.171)] was found between the risk of pancreatic cancer and serum TC.

CONCLUSION: Dietary cholesterol may be associated with an increased risk of pancreatic cancer in worldwide populations, except for Europeans. The results need to be confirmed further.

Key words: Dietary cholesterol; Serum total cholesterol; Pancreatic cancer; Risk; Meta-analysis

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Abstract

AIM: To evaluate the effect of dietary cholesterol and serum total cholesterol (TC) on the risk of pancreatic cancer.

METHODS: A literature search was performed up to June 2014 in PubMed, EMBASE, China National Knowledge Infrastructure and China Biology Medical

Core tip: Many epidemiological studies have explored the association of cholesterol with the risk of pancreatic cancer, but the results of these studies are conflicting. We conducted the current meta-analysis to evaluate the effect of dietary cholesterol and serum total cholesterol on the risk of pancreatic cancer. The results suggested that dietary cholesterol may be associated

with an increased risk of pancreatic cancer. However, the finding needs to be confirmed further.

Wang J, Wang WJ, Zhai L, Zhang DF. Association of cholesterol with risk of pancreatic cancer: A meta-analysis. *World J Gastroenterol* 2015; 21(12): 3711-3719 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3711.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3711>

INTRODUCTION

Pancreatic cancer is an uncommon but fatal malignant tumor. The overall 5-year survival rate of pancreatic cancer is less than 4%^[1]. Worldwide, the estimated numbers of cases and deaths for pancreatic cancer are 277000 and 266000 in 2008^[2], respectively. In the United States, the estimated numbers of new pancreatic cancer cases and deaths are 46420 and 39590 in 2014^[3], respectively. Several factors have been associated with the risk of pancreatic cancer, such as age^[4], body mass index (BMI)^[5], smoking^[6], coffee drinking^[7], hepatitis B virus (HBV) and hepatitis C virus (HCV) infection^[8], type 2 diabetes mellitus^[9] and family history^[10]. In addition, many nutritional factors, such as folate^[11], fat^[12] and cholesterol^[13-16], might also have an influence on the risk of pancreatic cancer.

Several epidemiologic studies have been performed to evaluate the relationship between cholesterol and the risk of pancreatic cancer. Although some studies found that dietary cholesterol was associated with an increased risk of pancreatic cancer^[13-15], others demonstrated no association between dietary cholesterol and the risk of pancreatic cancer^[17-19]. The association between serum total cholesterol (TC) and the risk of pancreatic cancer also remains controversial^[16,20,21]. So far, there is no sufficient epidemiological evidence to establish an association between the risk of pancreatic cancer and dietary cholesterol or serum TC level.

Therefore, we conducted a meta-analysis to evaluate the effect of dietary cholesterol and serum TC on the risk of pancreatic cancer.

MATERIALS AND METHODS

Search strategy

A literature search was performed up to June 2014 for relevant available articles published in English or Chinese from the following databases: (1) PubMed; (2) EMBASE; (3) China National Knowledge Infrastructure (CNKI); and (4) China Biology Medical literature database (CBM). The following search terms were used: "pancreatic cancer OR pancreatic neoplasm OR pancreatic carcinoma OR pancreatic tumour" and "cholesterol OR hypercholesterolemia". Moreover,

we reviewed the bibliographies of included articles to search additional studies not captured by our databases. The detailed steps of the literature search are shown in Figure 1.

Inclusion criteria

The inclusion criteria were as follows: (1) an observational study published as an original study to evaluate the association between the risk of pancreatic cancer and dietary cholesterol and serum TC; (2) the exposure of interest was cholesterol; (3) the outcome of interest was pancreatic cancer; and (4) relative risk (RR) and 95% confidence interval (CI) (or data to calculate these) were provided. The most recent and complete study was included if data from the same population had been published repeatedly.

Two investigators (JW and LZ) searched and reviewed all identified studies independently. If the two investigators cannot reach an agreement, it was resolved by consensus with a third reviewer.

Data extraction

The following data were extracted from each study by two investigators (JW and LZ) independently: the first author's name, publication year, country where the study was performed, study design, sample size and number of cases, mean age, male percentage in case (exposed) and control (unexposed) groups, RRs (we presented all results as RR for simplicity) with corresponding 95%CIs for highest vs lowest categories of cholesterol, the cut-points for cholesterol exposure and variables adjusted for in the analysis. We extracted the RRs that were adjusted for the most confounders.

Statistical analysis

Pooled measure was calculated as the inverse variance-weighted mean of the logarithm of RR with 95%CI to assess the strength of association between cholesterol and the risk of pancreatic cancer. The I^2 was adopted to assess the heterogeneity between studies (I^2 values of 0%, 25%, 50% and 75% represent no, low, moderate and high heterogeneity^[22], respectively). The random-effects model (REM) was used as the pooling method. Meta-regression was performed to evaluate the potentially important covariates that might exert substantial impacts on between-study heterogeneity^[23]. Influence analysis was performed with one study removed at a time to assess whether the results could have been affected markedly by a single study^[24]. The Egger *et al.*^[25] regression asymmetry test and the funnel plot were adopted to evaluate publication bias. Subgroup analysis was performed by study design (case-control or cohort study) and continent (North America, Europe or others).

All statistical analyses were performed with STATA version 10.0 (Stata Corporation, College Station, TX, United States). All reported probabilities (*P*-values)

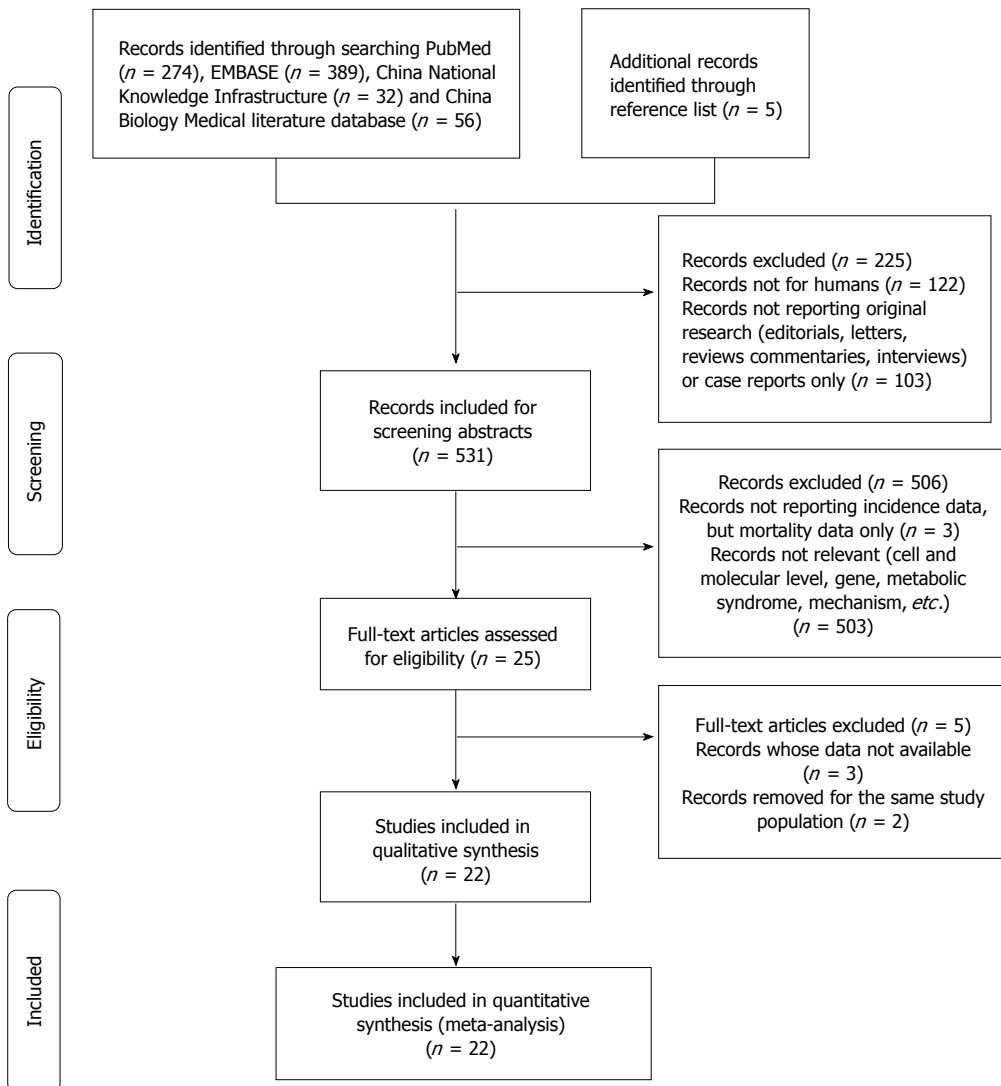


Figure 1 Flow diagram of literature search.

were two-sided with a statistical significance level of 0.05.

RESULTS

Study characteristics

For dietary cholesterol, 14 articles^[13-15,17-19,26-33] with 14 studies (4 cohort studies and 10 case-control studies) were included, involving 439355 participants. For serum TC, 6 articles^[16,20,21,34-36] with 8 studies (6 cohort studies and 2 case-control studies) were included, involving 1805697 participants. The detailed characteristics of the included studies are shown in Tables 1 and 2.

Quantitative synthesis

The main results are summarized in Table 3.

Dietary cholesterol and the risk of pancreatic cancer: For the highest vs lowest category of dietary cholesterol, the pooled RR of pancreatic cancer was

1.308 (95%CI: 1.097-1.559, $I^2 = 55.3\%$, $P_{\text{heterogeneity}} = 0.006$). The pooled RRs for case-control and cohort studies were 1.523 (95%CI: 1.226-1.893, $I^2 = 49.7\%$, $P_{\text{heterogeneity}} = 0.037$) and 1.023 (95%CI: 0.871-1.200, $I^2 = 0.0\%$, $P_{\text{heterogeneity}} = 0.508$), respectively. The pooled RRs for studies conducted in North America, Europe and others were 1.275 (95%CI: 1.058-1.537, $I^2 = 29.3\%$, $P_{\text{heterogeneity}} = 0.215$), 1.149 (95%CI: 0.863-1.531, $I^2 = 55.4\%$, $P_{\text{heterogeneity}} = 0.047$) and 2.495 (95%CI: 1.565-3.977, $I^2 = 0.0\%$, $P_{\text{heterogeneity}} = 0.362$), respectively (Figure 2).

Serum TC and the risk of pancreatic cancer: Serum TC level (highest vs lowest) was not significantly associated with the risk of pancreatic cancer (RR = 1.003, 95%CI: 0.859-1.171, $I^2 = 55.5\%$, $P_{\text{heterogeneity}} = 0.028$). The pooled RRs for European and Asian populations were 1.034 (95%CI: 0.722-1.481, $I^2 = 65.1\%$, $P_{\text{heterogeneity}} = 0.035$) and 1.005 (95%CI: 0.847-1.192, $I^2 = 56.2\%$, $P_{\text{heterogeneity}} = 0.077$), respectively.

Table 1 Characteristics of studies for dietary cholesterol included in the meta-analysis

Ref.	Country (year)	Study design	Mean age (case/control) Percentage of males (case/control)	Sample size (cases)	Cur-points for cholesterol exposure RR (95%CI)	Adjustment for covariates
Lin <i>et al</i> ^[3]	Japan -2005	Case-control	64.7/65.1 NA	327 -109	Dietary cholesterol exposure (mg), < 206 (referent), 206-330, > 330 [2.06 (1.11-3.85)]	Age and pack-years of smoking
Chan <i>et al</i> ^[14]	United States -2007	Case-control	NA	2233 -532	Dietary cholesterol exposure (g/d) median, 122.8 (referent), 192.6, 257.6, 368.9 [1.5 (1.1-2.0)]	Age, sex, BMI, race, education, smoking, history of diabetes and energy intake
Hu J <i>et al</i> ^[15]	Canada -2012	Case-control	54.7/51.9 61.6/57.1 56.2/50.5	5667 -628	Dietary cholesterol cut-point (mg/wk) < 966.261 (referent) 966.262-1412.753, 1412.754-1880.265, > 1880.266 [1.57 (1.09-2.26)]	Age, sex, BMI, province, education, alcohol drinking, pack year smoking, total of vegetable and fruit intake, saturated fat and total energy intake
Howe <i>et al</i> ^[17]	Metropolitan Toronto -1990	Case-control	64.6/64.8 56.6/53.5	754 -249	Mean difference per day quartile 4-quartile 1 [569 mg] [0.95 (0.51-1.75)]	Caloric and fibre intake, lifetime cigarette consumption
Bueno de Mesquita <i>et al</i> ^[18]	Netherlands -1991	Case-control	NA 54.9/48.3	644 -164	Dietary cholesterol [1.33 (0.72-2.45)]	Age, sex, response status, total smoking and dietary intake of energy
Lucenteforte <i>et al</i> ^[19]	Italy -2010	Case-control	NA 53.4/53.4	978 -326	First quintile of cholesterol exposure (referent), second <i>vs</i> first, third <i>vs</i> first, fourth <i>vs</i> first, fifth <i>vs</i> first [1.10 (0.68-1.77)]	Year of interview, education, tobacco smoking, history of diabetes and total energy intake
Baghurst <i>et al</i> ^[26]	Australia -1991	Case-control	NA 50.0/56.1	357 -104	First quintile of cholesterol exposure (referent), second <i>vs</i> first, third <i>vs</i> first, fourth <i>vs</i> first [3.19 (1.58-6.47)]	Age and pack-years of smoking
Chadrian <i>et al</i> ^[27]	Canada -1995	Case-control	63.9/62.1 54.2/51.5	418 -179	First quintile of cholesterol exposure (referent), second <i>vs</i> first, third <i>vs</i> first, fourth <i>vs</i> first [2.24 (0.83-6.05)]	Age, sex, lifetime cigarette consumption, response status and total energy intake
Heinen <i>et al</i> ^[28]	The Netherlands -2009	Case-cohort	NA 52.9/49.1	120852 -350	Dietary cholesterol (mg/d), first quintile of cholesterol exposure (referent), second <i>vs</i> first, third <i>vs</i> first, fourth <i>vs</i> first, fifth <i>vs</i> first [0.78 (0.52-1.18)]	Age, sex, BMI, energy, smoking, alcohol, history of diabetes mellitus, history of hypertension, vegetables and fruits intake
Kalapoathaki <i>et al</i> ^[29]	Greece -1993	Case-control	NA NA	362 -181	Dietary cholesterol (mg), an increment of about one standard deviation of the energy-adjusted residual of the corresponding nutritional variable [1.19 (0.96-1.47)]	Age, sex, hospital, past residence, years of schooling, smoking, diabetes mellitus and energy intake
Michaud <i>et al</i> ^[30]	United States -2003	Cohort	NA NA	88802 -178	Median of cholesterol exposure (g/d) 212 (referent), 275, 322, 371, 466 [1.11 (0.67-1.83)]	Pack-years of smoking, BMI, history of diabetes mellitus, caloric intake, height, physical activity, menopausal status and glycemic load intake
Nöthlings <i>et al</i> ^[31]	Hawaii and Los Angeles -2005	Cohort	65/60 51.2/45.3	190545 -482	Cholesterol density (mg/1000 kcal per day) median intake 56.8 (referent), 81.6, 100.4, 120.8, 156.8 [1.09 (0.89-1.32)]	Age, ethnicity, history of diabetes mellitus, familial history of pancreatic cancer, smoking status and energy intake
Stolzenberg-Solomon <i>et al</i> ^[32]	Finland -2002	Cohort	58/57 NA	27111 -163	First quintile of cholesterol exposure (referent), second <i>vs</i> first, third <i>vs</i> first, fourth <i>vs</i> first, fifth <i>vs</i> first [0.92 (0.53-1.59)]	Energy intake, age, years of smoking and energy-adjusted saturated fat intake
Zatonski <i>et al</i> ^[33]	Poland -1991	Case-control	62.2/63.2 61.8/45.6	305 -110	First quintile of cholesterol exposure (referent), second <i>vs</i> first, third <i>vs</i> first, fourth <i>vs</i> first [4.31 (1.60-11.59)]	Cigarette lifetime consumption and calories

NA: Not available; BMI: Body mass index.

Table 2 Characteristics of studies for serum total cholesterol included in the meta-analysis

Ref.	Country (year)	Study design	Mean age (case/control) Percentage of males (case/control)	Sample size (cases)	Cut-points for cholesterol Exposure RR (95%CI)	Adjustment for covariates
Wu <i>et al.</i> ^[36]	China (2012)	Case-control	59.3/59.3 58.6/58.6	840 (210)	Serum TC < 5.70 mmol/L (referent), ≥ 5.70 mmol/L [1.793 (1.067-3.013)]	Age, sex, hypertension, HBV markers, the levels of HDL, LDL, Tri and Apo B
Stolzenberg-Solomon <i>et al.</i> ^[20]	Finland (2002)	Cohort	NA	29048 (172)	Serum TC < 5.18 mmol/L (referent), ≥ 5.18 mmol/L [0.88 (0.60-1.28)]	Age, years smoked, cigarettes smoked per day, self-reported history of diabetes and bronchial asthma, occupational activity and measured high blood pressure
Johansen <i>et al.</i> ^[21]	Austria, Norway, and Sweden (2010)	Cohort	NA	289866 (543)	Serum TC mean level (mmol/L) 4.5 (referent), 5.3, 5.8, 6.4, 7.6 [0.70 (0.53-0.93)]	Age, BMI and smoking status
Johansen <i>et al.</i> ^[21]	Austria, Norway, and Sweden (2010)	Cohort	NA	288834 (314)	Serum TC mean level (mmol/L) 4.4 (referent), 5.1, 5.7, 6.3, 1.11 [0.75 (0.53-1.64)]	Age, BMI and smoking status
Kitahara <i>et al.</i> ^[34]	South Korea (2011)	Cohort	NA	756604 (1799)	Serum TC (mg/dL) < 160 (referent), 160-179, 180-199, 200-239, ≥ 240 [0.88 (0.74-1.05)]	Smoking, drinking, fasting serum glucose, BMI, hypertension and physical activity
Kitahara <i>et al.</i> ^[34]	South Korea (2011)	Cohort	NA	433115 (776)	Serum TC (mg/dL) < 160 (referent), 160-179, 180-199, 200-239, ≥ 240 [0.96 (0.74-1.24)]	Smoking, drinking, fasting serum glucose, BMI, hypertension and physical activity
Kuzmickiene <i>et al.</i> ^[35]	Lithuania (2013)	Cohort	NA	6788 (73)	Serum TC (mmol/L) < 5.20 (referent), 5.20-5.89, 5.90-6.62, ≥ 6.63 [1.76 (0.87-3.55)]	Age, BMI, smoking status, alcohol consumption and education
Xu <i>et al.</i> ^[36]	China (2011)	Case-control	61.4/60.74 59.3/60.5	602 (290)	Serum TC (mmol/L) < 5.72 (referent), ≥ 5.72 [1.01 (0.88-1.17)]	Diabetes mellitus, smoking, hypertension, family history of cancer, history of gastrointestinal surgery, history of biliary disease, history of chronic pancreatitis and triglyceride

NA: Not available; BMI: Body mass index.

Sources of heterogeneity and sensitivity analysis

In order to explore the between-study heterogeneity, we performed univariate meta-regression with the covariates of sex, age, publication year, sample size, continent where the study was conducted and study design. For the analysis between the risk of pancreatic cancer and dietary cholesterol, study design was found to contribute significantly to the between-study heterogeneity ($P = 0.037$). After excluding two studies^[26,33] ($RR > 3.0$), the heterogeneity was reduced to 29.4% ($P_{\text{heterogeneity}} = 0.158$), and the pooled RR was 1.204 (95%CI: 1.050-1.380). For the analysis between the risk of pancreatic cancer and serum TC, no covariate contributed significantly to the between-study heterogeneity.

Influence analysis

For the relationship between dietary cholesterol and the risk of pancreatic cancer, the summary RR (95%CI) ranged from 1.203 (95%CI: 1.079-1.341) to 1.291 (95%CI: 1.146-1.455) in influence analysis (Figure 3). For the relationship between serum TC and the risk of pancreatic cancer, the range was from 0.941 (95%CI: 0.840-1.054) to 1.003 (95%CI: 0.913-1.101).

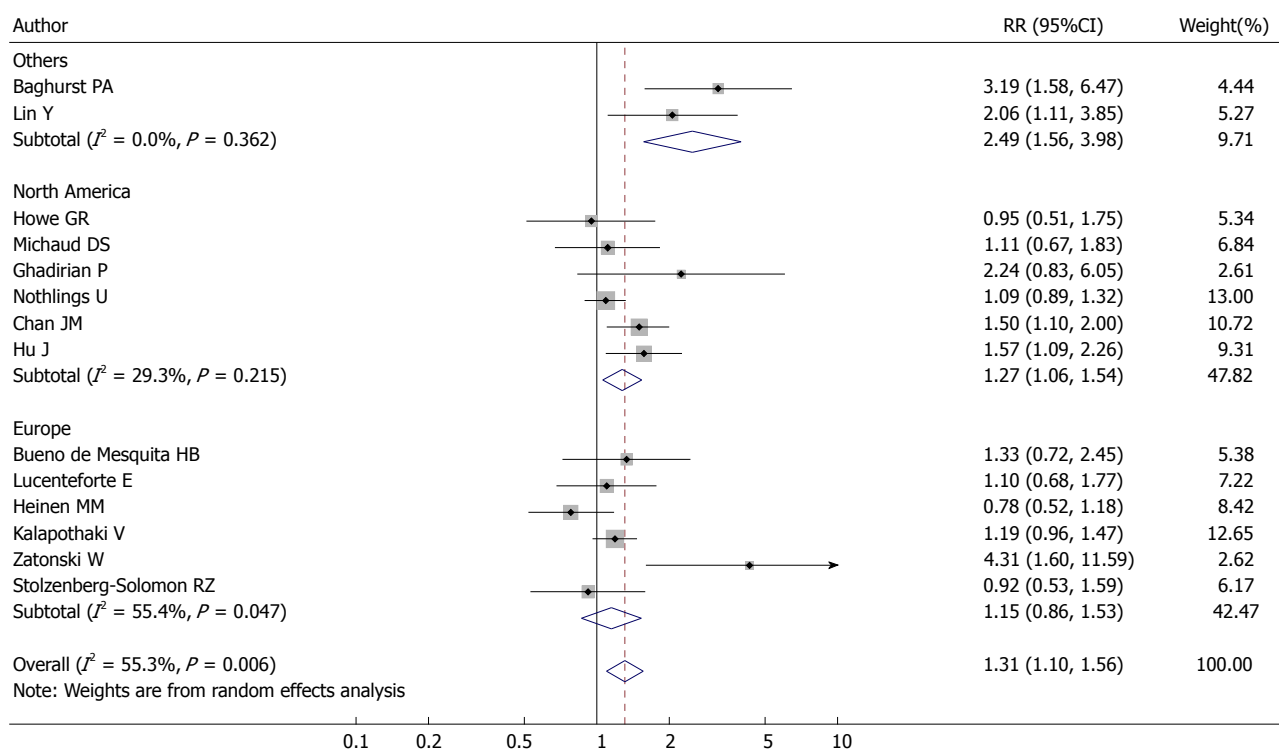
Publication bias

Egger test and funnel plot showed no evidence of significant publication bias for the analysis between the risk of pancreatic cancer and dietary cholesterol ($P = 0.107$) (Figure 4) or serum TC ($P = 0.204$).

Table 3 Pooled relative risks of associations between pancreatic cancer and dietary cholesterol and serum total cholesterol

Cholesterol source	Subgroup	No. of studies	Pooled RR (95%CI) REM	I^2	$P_{\text{heterogeneity}}$
Dietary cholesterol	All studies	14	1.308 (1.097-1.559)	55.3%	0.006
	After excluding two studies ^[24,31] (RR > 3.0)	12	1.204 (1.050-1.380)	29.4%	0.158
	Study design				
	Case-control	10	1.523 (1.226-1.893)	49.7%	0.037
	Cohort	4	1.023 (0.871-1.200)	0.0%	0.508
	Continent				
	North America	6	1.275 (1.058-1.537)	29.3%	0.215
Serum TC	Europe	6	1.149 (0.863-1.531)	55.4%	0.047
	Others	2	2.495 (1.565-3.977)	0.0%	0.362
	All studies	8	1.003 (0.859-1.171)	55.5%	0.028
	Continent				
	Europe	4	1.034 (0.722-1.481)	65.1%	0.035
	Asia	4	1.005 (0.847-1.192)	56.2%	0.077

TC: Total cholesterol; REM: Random effect model.

**Figure 2** Forest plot of the relative risks of studies on dietary cholesterol and pancreatic cancer.

DISCUSSION

Recently, many studies have been performed to evaluate the association between cholesterol and the risk of pancreatic cancer. However, the results are conflicting. Generally, individual study has a relatively small sample size with insufficient power to detect the effect. Therefore, we conducted a meta-analysis to get a more reasonable conclusion. This meta-analysis, containing 439355 participants for dietary cholesterol and 1805697 participants for serum TC, can effectively assess the association of cholesterol and the risk of pancreatic cancer. Findings from this meta-analysis suggested that dietary cholesterol may be associated with an increased risk of pancreatic

cancer. The association of dietary cholesterol with the risk of pancreatic cancer was significant in case-control studies, and for studies conducted in North America and others but not in Europe. No significant association between the risk of pancreatic cancer and serum TC was found in this meta-analysis.

The exact mechanism whereby high total cholesterol levels could lead to an increased risk of pancreatic cancer is unclear. There are several theories explaining the possible role of cholesterol in pancreatic cancer. Increased level of serum TC is related to increased levels of proinflammatory cytokines^[37-39]. Longstanding pre-existing chronic pancreatitis is a strong risk factor for pancreatic cancer^[40]. Moreover, dietary cholesterol may affect bile excretion. This may cause bile reflux

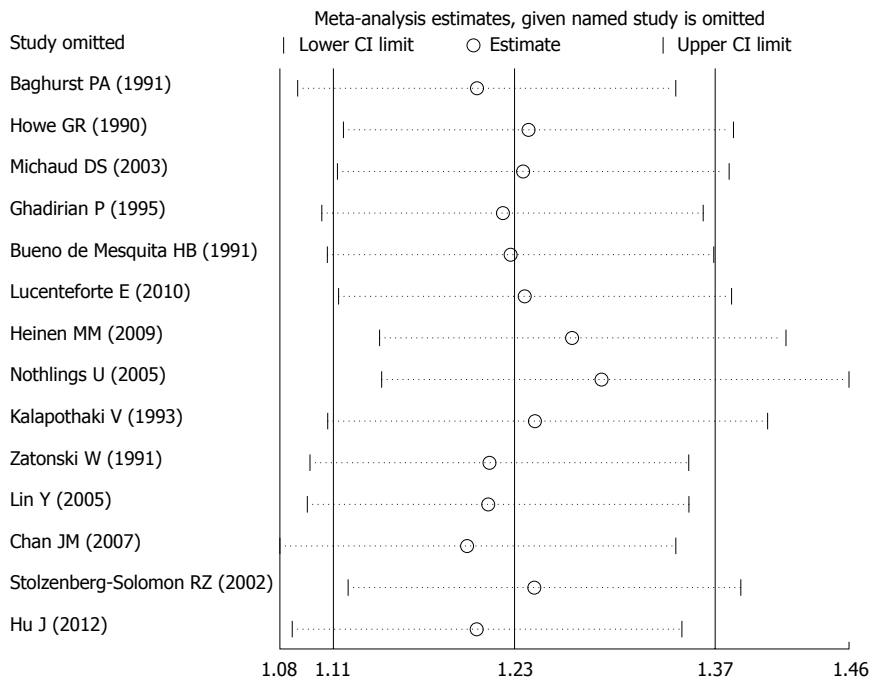


Figure 3 Influence analysis of individual study on the pooled estimate for studies on dietary cholesterol and pancreatic cancer.

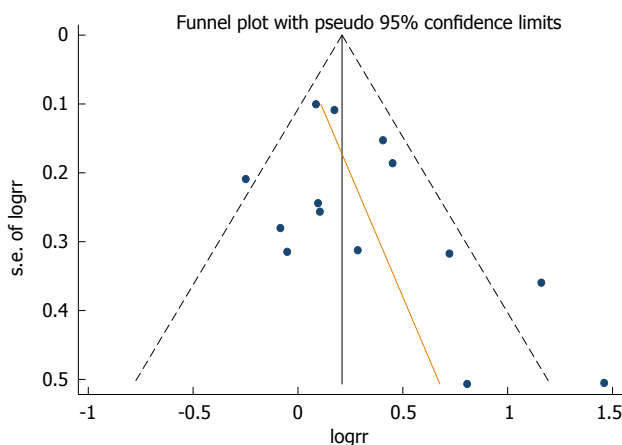


Figure 4 Funnel plot of the relative risks of 14 studies on dietary cholesterol and pancreatic cancer.

into the head of the pancreas *via* the common duct, where most tumors occur^[26,41].

Between-study heterogeneity is common in meta-analysis. It is essential to explore the potential sources of between-study heterogeneity. Diversity in a number of indeterminate characteristics such as sex, age, publication year, sample size, the continent where the study was performed or study design might be the source of between-study heterogeneity. Therefore, we explored the potential sources of the between-study heterogeneity with meta-regression. However, only study design was found to contribute to the between-study heterogeneity significantly in the analysis for dietary cholesterol. In subgroup analysis by study design, the between-study heterogeneities for case-control studies and cohort studies were reduced to

49.7% and 0.0%, respectively. After excluding two studies^[26,33] ($RR > 3.0$) in the analysis for dietary cholesterol, the between-study heterogeneity was reduced to 29.4%, and the result did not change substantially, suggesting that the result was stable.

This meta-analysis has several strengths. First, a large number of participants were included, allowing a much greater possibility of reaching a reasonable conclusion. Second, almost all studies included in this meta-analysis were adjusted for major risk factors, such as age, sex, smoking, BMI, energy intake, making the results more credible. Third, influence analysis showed that no individual study had an excessive influence on the pooled effects of dietary cholesterol and serum TC on the risk of pancreatic cancer. Fourth, after excluding two studies^[26,33] ($RR > 3.0$) in dietary cholesterol analysis, the between-study heterogeneity was reduced to 29.4%, but the result did not change substantially.

However, the present study has several limitations. First, unknown confounders might result in exaggerating or underestimating the risk. Second, disparate results were found between the association of dietary cholesterol and serum TC with the risk of pancreatic cancer. Third, in subgroup analysis by continent, a significant association between dietary cholesterol and the risk of pancreatic cancer was found for studies conducted in North America and others, but no association was found for those in Europe. However, the discrepancy might also be caused by the relatively small number of studies in each subgroup analysis. Fourth, results from case-control studies are susceptible to recall bias, thus prospective cohort studies that do not suffer from recall bias are believed

to provide better evidence. However, only 4 cohort studies were included in this meta-analysis. Therefore, further cohort studies are warranted to confirm this association. In addition, patients might change their dietary habits after the diagnosis of pancreatic cancer; however, in most case-control studies included in this meta-analysis, the investigators collected the dietary information of participants at least 1 year before the interview. Finally, although serum TC was not found to be associated with the risk of pancreatic cancer, the blood of patients was collected after the diagnosis of pancreatic cancer in case-control studies and at the start of the study in cohort studies.

In summary, this meta-analysis suggested that dietary cholesterol may be associated with the risk of pancreatic cancer in worldwide populations, except for Europeans. The finding needs to be confirmed further.

COMMENTS

Background

Pancreatic cancer is an uncommon but fatal malignant tumor. Several factors have been associated with the risk of pancreatic cancer, but the association between cholesterol and the risk of pancreatic cancer is still unclear.

Research frontiers

Until now, many epidemiological studies have explored the association of cholesterol with the risk of pancreatic cancer, but the results of these studies are conflicting.

Innovations and breakthroughs

This is the first meta-analysis to investigate the association of cholesterol with the risk of pancreatic cancer. Dietary cholesterol may be associated with an increased risk of pancreatic cancer in worldwide populations, except for Europeans.

Applications

The results of our study may give people instructions to prevent pancreatic cancer by limiting cholesterol intake.

Peer-review

This manuscript presents a well-designed meta-analysis that assessed the association between cholesterol and the risk of pancreatic cancer. The results suggest that dietary cholesterol may be associated with an increased risk of pancreatic cancer in worldwide populations, except for Europeans.

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Effects of neutrophil elastase inhibitor in patients undergoing esophagectomy: A systematic review and meta-analysis

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comparative studies were considered eligible for inclusion. Three reviewers evaluated the methodological quality of the included studies, and Stata 12.0 software was used to analyze the extracted data. The risk ratio (RR) was used to express the effect size of dichotomous outcomes, and mean difference (MD) or standardized mean difference was used to express the effect size of continuous outcomes.

RESULTS: Thirteen studies were included in this systematic review and nine studies were included in the meta-analysis. The duration of mechanical ventilation was significantly decreased in the sivelestat group on postoperative day 5 [$I^2 = 76.3\%$, SMD = -1.41, 95%CI: -2.63-(-0.19)]. Sivelestat greatly lowered the incidence of acute lung injury in patients after surgery ($I^2 = 0\%$, RR = 0.27, 95%CI: 0.08-0.93). However, it did not decrease the incidence of pneumonia, intensive care unit stay or postoperative hospital stay, and did not increase the incidence of complications such as anastomotic leakage, recurrent nerve palsy, wound infection, sepsis and catheter-related fever.

CONCLUSION: A neutrophil elastase inhibitor is beneficial in patients undergoing esophagectomy. More high quality, large sample, multi-center and randomized controlled trials are needed to validate this effect.

Key words: Neutrophil elastase inhibitor; Esophageal cancer; Esophagectomy; Systematic review; Meta-analysis

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Abstract

AIM: To evaluate the benefit and safety of sivelestat (a neutrophil elastase inhibitor) administration in patients undergoing esophagectomy.

METHODS: Online databases including PubMed, EMBASE, the Cochrane Library, Web of Knowledge, and Chinese databases (Wanfang database, VIP and CNKI) were searched systematically up to November 2013. Randomized controlled trials and high-quality

Core tip: Radical esophagectomy has been adopted in patients with esophageal carcinoma to improve survival. This technique is highly invasive, leading to excess surgical stress, a perioperative mortality of 3%-10%, and pulmonary disorders account for nearly 30%-60%. Sivelestat sodium hydrate, a specific

neutrophil elastase inhibitor, actively protects patients with acute respiratory diseases. The efficacy and safety of sivelestat administered during esophagectomy has produced conflicting results and the conclusions from relevant studies are presented. This meta-analysis revealed that sivelestat is beneficial in patients undergoing esophagectomy, especially in terms of the duration of mechanical ventilation and the incidence of pulmonary complications.

Wang ZQ, Chen LQ, Yuan Y, Wang WP, Niu ZX, Yang YS, Cai J. Effects of neutrophil elastase inhibitor in patients undergoing esophagectomy: A systematic review and meta-analysis. *World J Gastroenterol* 2015; 21(12): 3720-3730 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3720.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3720>

INTRODUCTION

Esophageal carcinoma is the sixth leading cause of cancer-related deaths worldwide, and its incidence is increasing rapidly^[1,2]. In recent years, multidisciplinary treatments have been adopted more and more frequently. Of these treatments, curative surgery remains the most important treatment option^[3,4]. Previous studies have shown that patients undergoing radical esophagectomy after neoadjuvant therapy achieved the highest long-term survival^[4-6].

Radical esophagectomy, which consists of video-assisted thoracoscopic esophagectomy, cervical esophagogastrostomy and two- or three-field lymph node dissection, is one of the most invasive surgical techniques performed in the gastrointestinal system^[7]. This excess surgical stress has led to a perioperative mortality rate of approximately 3%-10%^[8,9], and is mainly caused by systemic inflammatory response syndrome (SIRS)-associated complications, of which pulmonary disorders account for approximately 30%-60%^[10].

The lung is the main target organ for overproduced cytokines in SIRS; thus, pneumonia, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) occur frequently in patients undergoing esophagectomy^[11,12]. Current studies have demonstrated that neutrophil elastase (NE), which is secreted by IL-8 induced mature neutrophils, could represent the severity of postoperative pulmonary disorders^[13]. In addition, Suda *et al.*^[14] stated that a drug that could relieve SIRS and control neutrophil function might improve the postoperative clinical course following transthoracic esophagectomy.

Sivelestat sodium hydrate, a synthetic NE inhibitor, can competitively inhibit NE activity and does not affect other proteases^[15]. A positive treatment effect was reported in many studies, and the Japanese Respiratory Society recommends sivelestat for the treatment of ALI in the Guidelines for Treatment of

ALI/ARDS^[16]. However, reports on the benefits of sivelestat administration during esophagectomy in patients with esophageal carcinoma have shown conflicting results^[17-19]. It is not known whether sivelestat can improve the postoperative clinical course, reduce lung function damage, and alter blood, cytokine and lung injury markers. Although some traditional reviews exist, the data from these reviews are not comprehensive and are insufficient. Therefore, we performed a systematic review and meta-analysis to evaluate the benefit and safety of sivelestat administration in patients undergoing esophagectomy.

MATERIALS AND METHODS

Literature search

Online databases, including PubMed, EMBASE, the Cochrane Library, Web of Knowledge, and Chinese databases (Wanfang database, VIP and CNKI) were searched systematically and comprehensively up to November 2013. In addition, clinicalTrials.gov and recent conferences were also searched. Search terms were "esophageal cancer OR esophagectomy" in combination with "neutrophil elastase inhibitor OR sivelestat OR sivelestat sodium OR freslestat" without limitation of publication year, status and language. Review articles were also scanned to identify relevant studies by reading the reference list.

Study selection

Randomized controlled trials and high-quality comparative studies were considered eligible for inclusion if: (1) the participants were esophageal carcinoma patients undergoing esophagectomy; (2) neutrophil elastase inhibitor was compared with placebo (saline); and (3) outcomes mainly included data on postoperative clinical course, oxygenation, blood and cytokines. Studies on patients undergoing other major surgeries were excluded. Quantitative data were not necessary for inclusion. According to the inclusion criteria, two reviewers independently reviewed the searched literature and any disagreement was resolved by discussion.

Data extraction and quality assessment

Data were extracted and a form, which was devised in advance, was completed. The following data were recorded: basic information (author, country and year of publication), characteristics (sex, age and arm), treatment protocol (case, sivelestat dosage and usage), surgical background (operative time, blood loss, surgical procedure), outcome measures [duration of mechanical ventilation, intensive care unit (ICU) stay, SIRS, postoperative hospital stay, and P/F ratio], and complications. Another two reviewers carried out the data extraction, and the results were then cross-checked. Disagreement was resolved by discussion.

Three reviewers evaluated the methodological

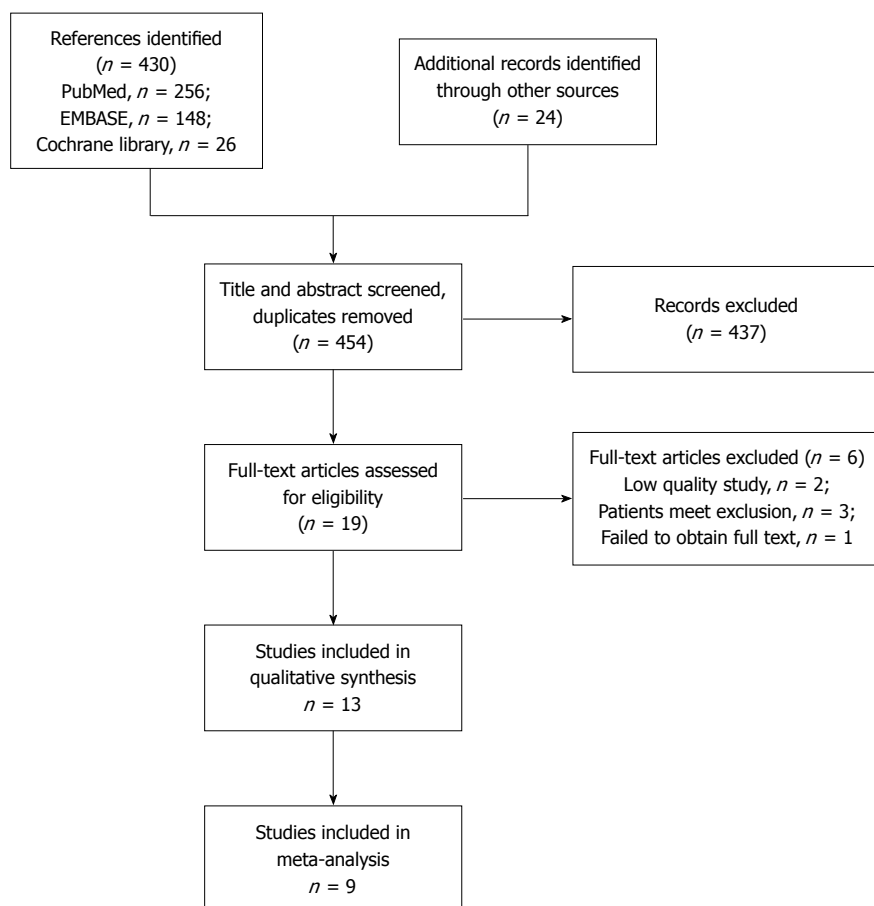


Figure 1 Flowchart of trials selection.

quality of the included studies according to the standard recommended by the Cochrane handbook^[20] for systematic reviews and meta-analyses. By studying the materials and methods section, quality assessment was performed by identifying the study type, randomization, blinding, allocation concealment, eligibility criteria, baseline comparability, participants lost to follow-up, ITT analysis, selective reporting, incomplete outcome and other biases.

Statistical analysis

Stata 12.0 software was used to analyze the extracted data. The risk ratio (RR) was used to express the effect size of dichotomous outcomes, and the mean difference (MD) or standardized mean difference was used to express the effect size of continuous outcomes. Cochran's *Q*-test and the I^2 statistic were used to estimate the heterogeneity among the pooled studies. If $P > 0.05$ or $I^2 < 50\%$, the heterogeneity was thought to be insignificant, and a fixed-effect model was adopted in the meta-analysis. If the heterogeneity was significant, a random-effect model was adopted and the source of heterogeneity was investigated using clinical and statistical aspects. In addition, sensitivity was assessed to judge the reliability of the evidence, and both Begg's test and Egger's test were conducted to determine publication bias.

This review was performed in accordance with The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. The present protocol has not been published or registered elsewhere.

RESULTS

Literature search

The flowchart of the trial selection is shown in Figure 1. A total of 454 references were identified from the online databases and other sources, and after screening the title and abstract, 17 references were selected for full-text assessment. In total, 13 studies were included in this systematic review^[14,19-30] and nine studies were included in the meta-analysis^[14,19,20,22,24-28].

Study characteristics and quality assessment

Tables 1 and 2 describe the baseline and basic information on the included studies. Ten studies had two arms: sivelestat-treated arm and saline-treated or control arm, and one study^[21] had three arms: two sivelestat-treated arms and a control arm. All the studies were performed in Japan, with 10 published in English and one published in Japanese. Other information, such as the sex and age of participants, dosage and usage of sivelestat, and surgical procedure related indices are summarized in detail,

Table 1 Basic characteristics of the included studies

Ref.	Year	Arm	Case (n)	Sex (M/F)	Age (yr)	Usage of sivelestat
Sato <i>et al</i> ^[19]	2001	SSH	8	-	63.9 ± 6.9	150000 U diluted in 20 mL normal saline every 12 h from operation to POD 5
		Saline	8	-	64.6 ± 8.7	
Akamoto <i>et al</i> ^[20]	2007	SSH	6	5/1	70.8 ± 5.5	4.8 mg/kg per day of sivelestat + 240 mL saline from operation to POD 3
		Saline	7	5/2	65.7 ± 2.9	
Kawahara <i>et al</i> ^[22]	2010	SSH	10	7/3	64 (50-78) ¹	300 mg/d of sivelestat + 200 mL saline from operation to POD 3
		Saline	10	10/0	63 (65-69)	
Makino <i>et al</i> ^[24]	2011	SSH	16	12/4	65 (61-68) ²	4.8 mg/kg per day of sivelestat + 240 mL saline from operation to POD 7
		Saline	15	13/2	66 (63-69)	
Yamaguchi <i>et al</i> ^[29]	2011	SSH	12	9/3	59 ± 5	0.2 mg/kg per hour sivelestat from operation to POD 1
		Saline	12	9/3	60 ± 8	
Iwahashi <i>et al</i> ^[21]	2011	Arm1	15	13/2	65 ± 8	Arm1: 0.2 mg/kg per hour sivelestat from operation to POD 1;
		Arm2	15	9/6	64 ± 7	Arm 2: 0.2 mg/kg per hour sivelestat from operation to POD 5
		Control	15	10/5	67 ± 8	0.2 mg/kg per hour sivelestat
Yamaki <i>et al</i> ^[30]	2005	SSH	9	-	62 ± 9	
		Control	6	-	69 ± 8	0.2 mg/kg per hour sivelestat after operation till POD 5
Ono <i>et al</i> ^[28]	2007	SSH	7	4/3	61 ± 12	
		Control	10	7/3	70 ± 7	0.2 mg/kg per hour sivelestat diluted with saline after operation till POD 6
Suda <i>et al</i> ^[14]	2007	SSH	18	15/3	60 (55-65) ³	
		Control	25	20/5	56 (52-66)	0.2 mg/kg per hour from operation and during mechanical ventilation support
Kobayashi <i>et al</i> ^[23]	2010	SSH	60	56/4	66 ± 7	
		Control	28	24/4	60 ± 10	0.2 mg/kg per hour sivelestat diluted with saline after operation till POD 5
Mimatsu <i>et al</i> ^[25]	2011	SSH	22	21/1	59 ± 11	
		Control	20	19/1	63 ± 9	0.2 mg/kg per hour sivelestat after operation till POD 3
Nishiyama <i>et al</i> ^[27]	2012	SSH	26	23/3	67 ± 8	
		Control	27	23/4	63 ± 8	0.2 mg/kg per hour sivelestat with 5% dextrose in water from operation till POD 3
Nagai <i>et al</i> ^[26]	2013	SSH	42	39/3	66 ± 9	
		Control	35	31/4	63 ± 8	

¹Range; ²95%CI; ³Inter-quartile range. SSH: Sivelestat sodium hydrate; POD: Postoperative day; Age is shown as mean ± SD.

Table 2 Basic surgical characteristics of patients in the included studies

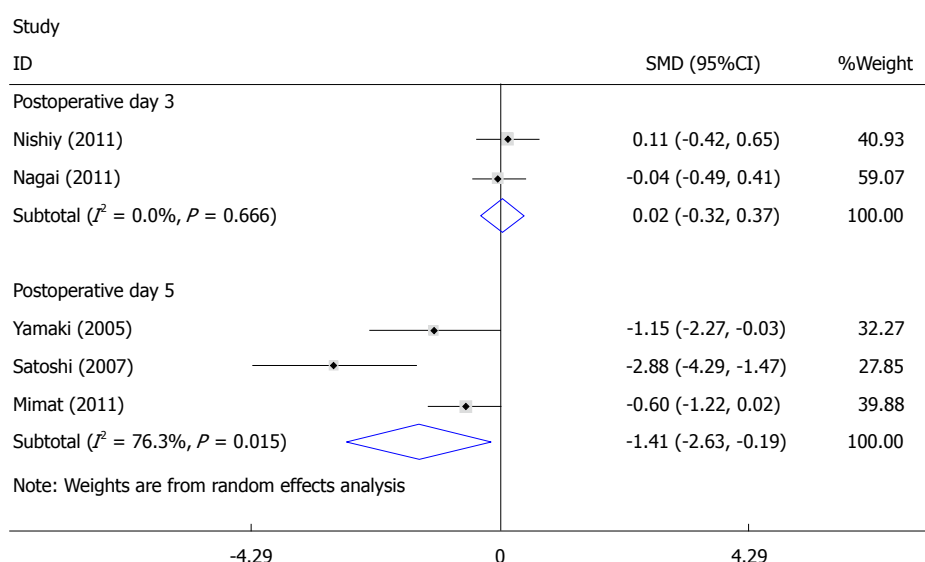
Ref.	Arm	Operative time (min)	Blood loss (mL)	Surgical procedure
Sato <i>et al</i> ^[19]	SSH	357 ± 58	615 ± 268	Extensive resection including lymph node dissection
	Saline	326 ± 23	712 ± 184	
Akamoto <i>et al</i> ^[20]	SSH	496 ± 140	1 672 ± 426	Esophagectomy and esophagogastric anastomosis
	Saline	569 ± 46	1 339 ± 316	
Kawahara <i>et al</i> ^[22]	SSH	517 (range 443-733)	305 (range 180-1050)	Video-assisted thoracoscopic oesophagectomy
	Saline	549 (range 453-785)	32 (range 150-1910)	
Makino <i>et al</i> ^[24]	SSH	433 (95%CI: 399-467)	468 (95%CI: 380-556)	Video-assisted thoracoscopic oesophagectomy
	Saline	431 (95%CI: 407-455)	514 (95%CI: 386-643)	
Yamaguchi <i>et al</i> ^[29]	SSH	387 ± 57	488 ± 229	Right-sided transthoracic esophagectomy with cervical esophagogastrotomy and lymph node dissection
	Saline	363 ± 85	376 ± 166	
Iwahashi <i>et al</i> ^[21]	SSH	491 ± 62	422 ± 210	Radical esophagectomy with a two- or three-field lymph node dissection <i>via</i> a cervicothoracoabdominal approach
	SSH	466 ± 72	405 ± 262	
	Control	482 ± 69	430 ± 173	
Yamaki <i>et al</i> ^[30]	SSH	538 ± 121	969 ± 505	Radical esophagectomy
	Control	552 ± 157	1134 ± 682	
Ono <i>et al</i> ^[28]	SSH	573.4 ± 72.6	1685.1 ± 1255.3	Esophagectomy and reconstruction with gastric mobilization by right posterolateral thoracotomy and laparotomy
	Control	568.7 ± 164.1	1032.4 ± 347.7	
Suda <i>et al</i> ^[14]	SSH	458 (95%CI: 373-545)	361 (95%CI: 218-682)	Transthoracic esophagectomy
	Control	626 (95%CI: 541-700)	520 (95%CI: 216-700)	
Kobayashi <i>et al</i> ^[23]	SSH	311 ± 66	359 ± 253	Thoracoscopy-assisted subtotal esophagectomy
	Control	412 ± 71	402 ± 161	
Mimatsu <i>et al</i> ^[25]	SSH	407.3 ± 74.6	346.7 ± 122.2	Transthoracic esophagectomy with reconstruction of the stomach role <i>via</i> the posterior sternum
	Control	396.7 ± 96.3	354.4 ± 134.5	
Nishiyama <i>et al</i> ^[27]	SSH	450.2 ± 64.1	813.6 ± 548.4	Thoracotomy total thoracic esophagectomy, chest wall-antral stomach reconstruction, and 3-regional lymph node dissection
	Control	445.8 ± 87.9	735.2 ± 479.0	
Nagai <i>et al</i> ^[26]	SSH	576.4 ± 126.7	630.1 ± 392.0	Subtotal esophagectomy and reconstruction through a right posterolateral thoracotomy and upper midline laparotomy
	Control	537.3 ± 120.2	494.2 ± 312.7	

SSH: Sivelestat sodium hydrate.

Table 3 Quality assessment of the included trials

Ref.	Type	Randomization	Blinding	Allocation concealment	Eligibility criteria	Baseline comparability	> 85% participants followed up	ITT analysis	Selective reporting	Incomplete outcome	Other bias
Sato <i>et al</i> ^[19]	RCT	M	U	U	Y	Y	Y	Y	U	N	U
Akamoto <i>et al</i> ^[20]	RCT	Y	Y, single blinding	U	Y	Y	Y	Y	U	N	U
Kawahara <i>et al</i> ^[22]	RCT	M	M, double blinding	U	Y	Y	Y	Y	U	N	U
Makino <i>et al</i> ^[24]	RCT	Y	Y, triple blinding	Y	Y	Y	Y	Y	U	N	U
Yamaguchi <i>et al</i> ^[29]	RCT	M	U	U	Y	Y	Y	Y	U	U	U
Iwahashi <i>et al</i> ^[21]	non-RCT	N	U	U	Y	Y	Y	N	U	N	U
Yamaki <i>et al</i> ^[30]	non-RCT	N	N	N	M	Y	Y	U	U	N	U
Ono <i>et al</i> ^[28]	non-RCT	N	N	N	Y	Y	Y	Y	U	N	U
Suda <i>et al</i> ^[14]	non-RCT	N	N	N	Y	Y	Y	Y	U	N	U
Kobayashi <i>et al</i> ^[23]	non-RCT	N	N	N	Y	Y	Y	Y	N	N	U
Mimatsu <i>et al</i> ^[25]	non-RCT	N	N	N	Y	Y	Y	Y	N	N	U
Nishiyama <i>et al</i> ^[27]	non-RCT	N	N	N	Y	Y	Y	Y	N	N	U
Nagai <i>et al</i> ^[26]	non-RCT	N	N	N	M	Y	Y	Y	U	N	U

M: Mentioned (the study just mentioned the item but without detailed description); Y: Yes (the study mentioned and detailed the item); N: No (the study did not report the item); U: Unclear.

**Figure 2** Duration of mechanical ventilation on postoperative days 3 and 5.

and all showed no significant differences between the treatment group and control group. Table 3 shows the results of the methodological quality assessment, which was carried out according to the methods recommended by The Cochrane Collaboration.

Mechanical ventilation

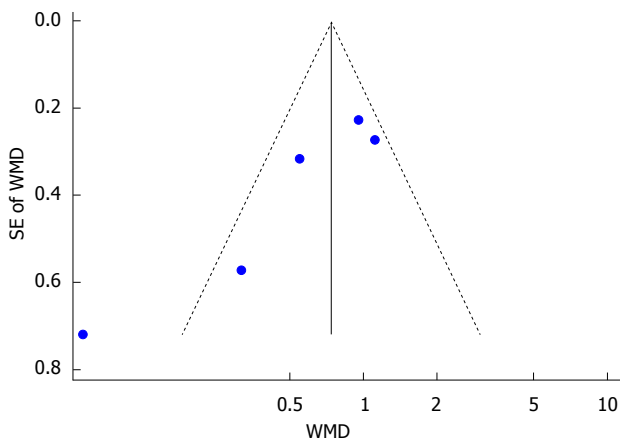
The duration of mechanical ventilation was reported in eight studies^[14,22,24-28,30], and five of these studies were pooled quantitatively in this meta-analysis^[25-28,30]. There was significant heterogeneity among the trials. To investigate the source of heterogeneity, according to postoperative day (POD) of sivelestat administration, subgroup analysis including POD

3 (sivelestat was administrated until POD3 and POD 5 (sivelestat was administrated until POD 5) was performed. When compared with the control group, the duration of mechanical ventilation was significantly decreased in the sivelestat group on POD 5 [$I^2 = 76.3\%$, SMD = -1.41, 95%CI: -2.63-(-0.19)]. Although the duration of mechanical ventilation was also decreased in the sivelestat group on POD 3, it failed to reach statistical significance ($I^2 = 0\%$, SMD = -0.68, 95%CI: -1.38-0.02). Begg's test and Egger's test showed that publication bias might exist ($P = 0.027$, 95%CI: -8.82-1.06). These data are shown in Figures 2 and 3. The data in the other three studies are summarized in Table 4.

Table 4 Summary of qualitative pooled data

Study	Kawah <i>et al</i> ¹		Makino <i>et al</i> ²		Suda <i>et al</i> ³	
	SSH <i>vs</i> control	P value	SSH <i>vs</i> control	P value	SSH <i>vs</i> control	P value
Mechanical ventilation	24.5 (24.3-28.7) <i>vs</i> 24.5 (23.9-49.1)	0.796	89.5 (57.3, 121.7) <i>vs</i> 204 (77.4, 330.6)	0.046	1 (1-1.5) <i>vs</i> 1.5 (1-2)	0.008
ICU stay	64.0 (39-109) <i>vs</i> 74.5 (39.0-109)	0.481	5.7 (4.1, 7.4) <i>vs</i> 8.8 (5.5, 12.1)	0.048	1.5 (1.5-1.9) <i>vs</i> 2.5 (1.5-3.5)	0.018
SIRS	17 (9-36) <i>vs</i> 49 (15-60)	0.009	2.8 (2.1, 3.6) <i>vs</i> 5.6 (4.2, 7.0)	0.001	3.5 (2-5.8) <i>vs</i> 5 (3.8-10.8)	0.026
Postoperative hospital stay	32 (19-46) <i>vs</i> 31 (18-81)	0.853	31.4 (23.8, 38.9) <i>vs</i> 37.1 (31.1, 43.1)	0.077		

¹Data is shown as the mean (range); ²Data is shown as the mean (95%CI); ³Data is shown as the median (inter-quartile range). SSH: Sivelestat sodium hydrate.

**Figure 3** Begg's and Egger's test of mechanical ventilation.

Pulmonary complications

Pneumonia and ALI were common pulmonary complications after esophagectomy. Seven studies reported data on pneumonia^[21,22,24-28], and the fixed effects meta-analysis showed that sivelestat decreased the incidence of pneumonia compared with the control; however, the difference was not statistically significant ($I^2 = 0\%$, $RR = 0.84$, 95%CI: 0.47-1.50). Available data on ALI was reported in two studies^[14,24]. The fixed effects model meta-analysis demonstrated that sivelestat greatly decreased the incidence of ALI in patients after surgery ($I^2 = 0\%$, $RR = 0.27$, 95%CI: 0.08-0.93). Begg's test and Egger's test indicated that no publication bias existed ($P = 0.214$, 95%CI: -3.24-0.87). These data are shown in Figures 4 and 5.

SIRS

Five studies presented data on SIRS^[14,22,24-26], and these studies demonstrated that sivelestat decreased the duration of SIRS. Of these five studies, four^[14,22,24,25] stated that there were significant differences between the sivelestat group and the control group ($P = 0.046$, $P = 0.048$, $P = 0.018$, $P = 0.048$), but one^[26] stated that the difference failed to reach statistical significance ($P > 0.05$), as shown in Table 4.

ICU stay

Six studies provided data on ICU stay^[14,22,24,26-28], and three of these studies were pooled quantitatively in the fixed effect analysis^[26-28]. The results showed that sivelestat decreased ICU stay, but this failed to achieve statistical significance [$I^2 = 0\%$, $SMD = -0.22$, 95%CI: -0.54-(-0.11)], as shown in Figure 6. In the other three studies, one study reported no statistically significant difference, and two studies found a statistically significant difference between the sivelestat group and the control group, as summarized in Table 4.

Postoperative hospital stay

Postoperative hospital stay was reported in four studies^[22,24,26,27], and two of these studies^[26,27] were pooled quantitatively in the fixed effect analysis. The results showed that sivelestat decreased postoperative hospital stay, but it failed to achieve statistical significance [$I^2 = 36.2\%$, $SMD = -0.27$, 95%CI: -0.63-(-0.09)], as shown in Figure 7. The other two studies^[22,24] showed no significant difference, as summarized in Table 4.

Other complications

With the exception of pulmonary complications, other complications such as anastomotic leakage, recurrent nerve palsy, wound infection and sepsis were also reported in the included studies. Fixed effects analysis demonstrated that no significant difference existed between the sivelestat group and the control group in terms of anastomotic leakage ($I^2 = 0\%$, $RR = 1.26$, 95%CI: 0.71-2.22), recurrent nerve palsy ($I^2 = 0\%$, $RR = 1.34$, 95%CI: 0.62-2.90), wound infection ($I^2 = 0\%$, $RR = 1.12$, 95%CI: 0.53-2.37), sepsis ($I^2 = 0\%$, $RR = 0.55$, 95%CI: 0.09-3.43) and catheter-related fever ($RR = 0.14$, 95%CI: 0.01-2.39). Overall, sivelestat did not significantly increase the incidence of these complications ($I^2 = 0\%$, $P = 1.10$, 95%CI: 0.75-1.59), and Begg's test and Egger's test indicated that no publication bias existed ($P = 0.53$, 95%CI: -1.57-0.84). These data are shown in Figures 8 and 9.

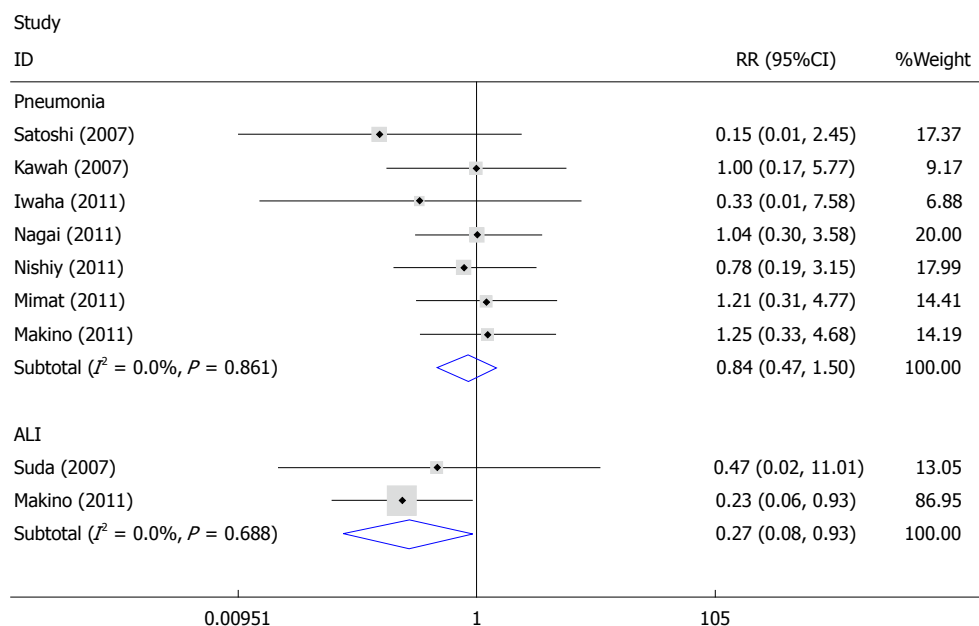


Figure 4 Pulmonary complications.

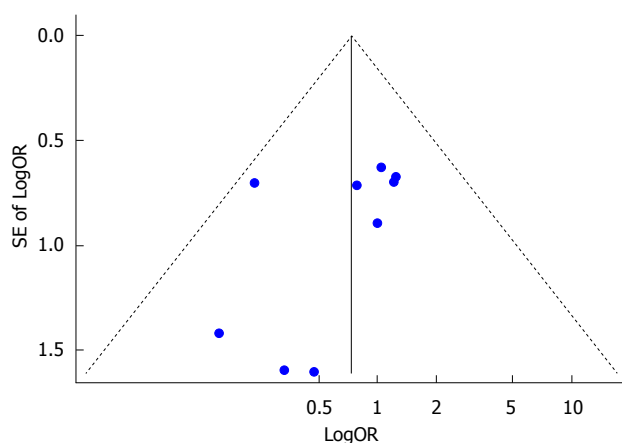


Figure 5 Begg's and Egger's test of pulmonary complications.

DISCUSSION

Some studies have found that patients undergoing esophagectomy benefit from methylprednisolone administration with no adverse effects. However, even when pre-operative methylprednisolone is administered, pulmonary complications frequently occur. This may be caused by the systemic inflammatory response following esophagectomy, leading to accumulation of neutrophils in the lungs. Subsequent local release of neutrophil elastase (NE) injures the lung^[18,31]. As glucocorticoids do not affect the release or function of NE, additional selective inhibition of NE might be beneficial. Indeed, the results of the meta-analysis showed that compared with the control group, the duration of mechanical ventilation support was reduced in the sivelestat group. Subgroup analysis demonstrated that this reduction in the duration of mechanical ventilation support failed to reach statistical significance in the sivelestat group

on POD 3, but it was significantly decreased in the sivelestat group on POD 5. Our study revealed that sivelestat administered at different times may lead to different clinical outcomes, and the administration of sivelestat should be continued up to at least POD 5 to decrease the time required for mechanical ventilation support.

Pneumonia and ALI are common pulmonary complications after esophagectomy^[10], and our results indicated that although sivelestat may not decrease the incidence of pneumonia compared with the control, it greatly reduced the incidence of ALI in patients after surgery. Although ARDS and SIRS have been clearly defined during the American-European consensus conferences, the criteria for pneumonia differ widely^[32]. Consequently, the study results for pneumonia should be considered with caution. Furthermore, pneumonia after esophagectomy can be caused by various factors such as increased infection, invasive surgical procedures, administration of methylprednisolone, decreased pulmonary function and immunity, and the use of mechanical ventilation support^[33]. ALI mainly occurs because of increased levels of cytokines in the serum, especially NE secreted by neutrophils. Thus, as a specific inhibitor of NE, sivelestat, had a very limited effect on postoperative pneumonia, but a very strong effect on postoperative ALI. In addition, sivelestat had a positive effect on pulmonary function. Kawaha *et al.*^[22] reported a significant increase in $\text{PaO}_2/\text{FiO}_2$ on POD 1 and 7; Suda *et al.*^[14] reported a significant increase in $\text{PaO}_2/\text{FiO}_2$ on POD 1; and Nishiyama *et al.*^[27] reported a significant increase in PaO_2 on POD 5.

Most studies reported a reduction in the duration of postoperative SIRS; however, two studies found no statistically significant difference^[21,26]. Of these two studies, Iwashashi *et al.*^[21] performed esophagectomy

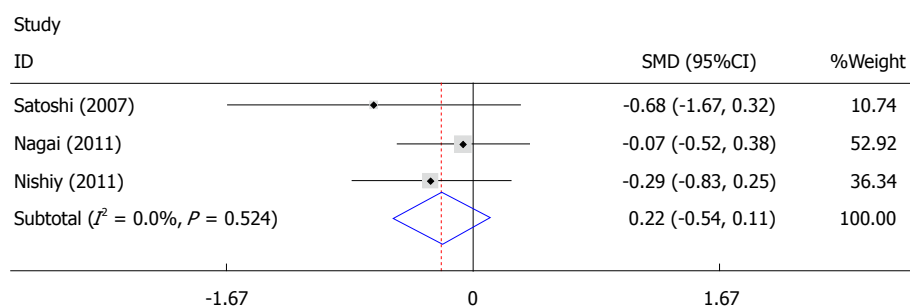


Figure 6 Intensive care unit stay.

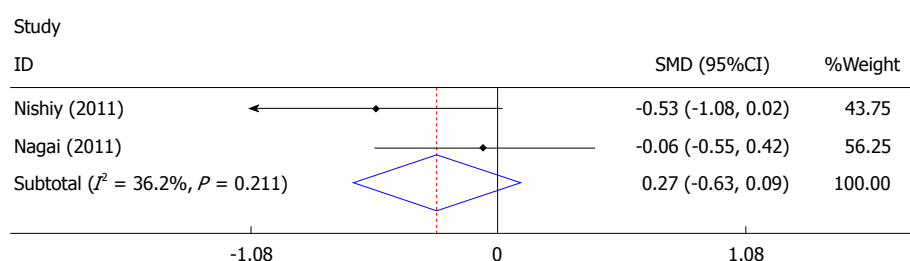


Figure 7 Postoperative hospital stay.

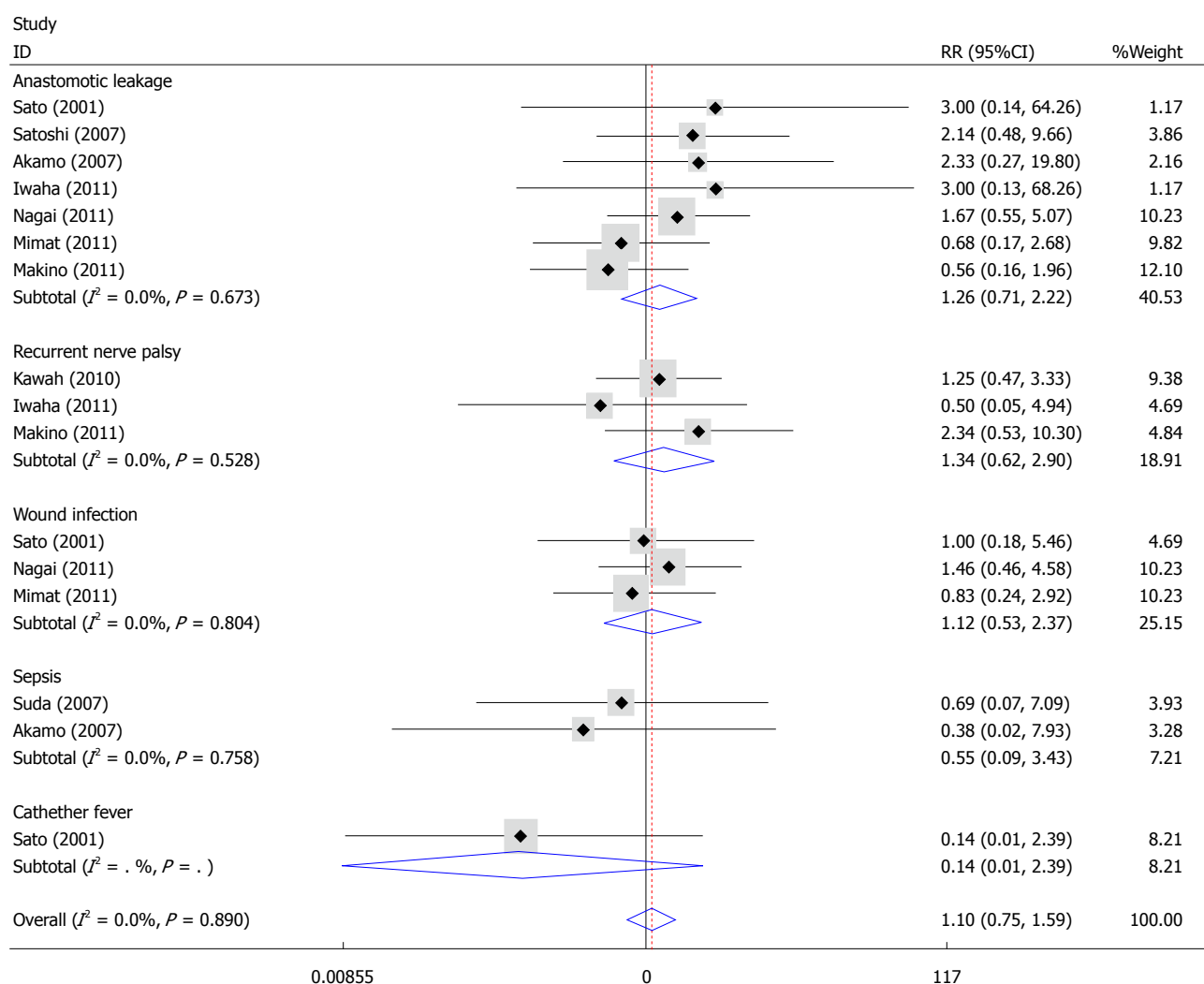


Figure 8 Other complications.

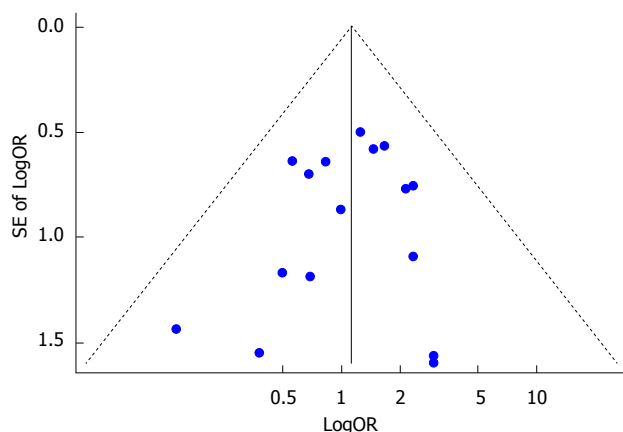


Figure 9 Begg's and Egger's test of other complications.

using the cervicothoracoabdominal approach, and Nagai *et al.*^[26] performed subtotal esophagectomy *via* a right posterolateral thoracotomy and upper midline laparotomy. Compared with current video-assisted thoracoscopic esophagectomy^[34,35], their surgical procedures appeared to be more invasive and led to more blood loss, which induced a more acute SIRS state. Therefore, additional sivelestat administration after the more invasive surgical procedure may have little clinical benefit, and the effects of different procedures in addition to higher dose of sivelestat should be investigated in the future.

The meta-analysis results showed that sivelestat may have decreased ICU stay; however, this decrease failed to achieve statistical significance. In the other three studies mentioned previously, two studies^[14,24] reported significant differences, while one study^[22] reported no significant difference, thus there is no consensus on ICU stay. Postoperative hospital stay was reported in four studies^[22,24,26,27], and only two of these studies^[26,27] were pooled quantitatively in the fixed effect analysis. The results showed that sivelestat might have decreased postoperative hospital stay; however, this decrease failed to achieve statistical significance. The other two studies^[22,24] showed no significant difference. With sivelestat administrated after surgery, the mechanical ventilation support, pulmonary complications and SIRS were improved; however, the ICU stay and postoperative hospital stay were not significantly shortened. Possible explanations for these findings are as follows: (1) limited number of studies included in the analysis; (2) insufficient data in the studies; (3) different protocols for discharging from the ICU and hospital adopted in the studies; (4) heterogeneity between the studies; and (5) different protocols of sivelestat administration.

One study performed a cost-analysis^[27], which showed that only surgery costs were significantly lower in the sivelestat group compared with the control group, and there were no significant differences in the hospitalization, medication or total costs. Therefore,

additional sivelestat did not increase medical costs. With regard to safety, our study demonstrated that sivelestat did not increase the risk of complications, including anastomotic leakage, recurrent nerve palsy, wound infection and sepsis.

There are also some weaknesses with the present evidence. Some of the included trials were non-RCTs, which may have increased the risk of random errors. Dissimilar procedures, such as minimally invasive or traditional surgery with different operative time and blood loss, could affect patient outcomes. In addition, different concentrations of sivelestat administered with inconsistent doses of methylprednisolone may decrease the risk of pulmonary complications. All of these factors suggest that there may be unavoidable bias in the pooled results, which in turn limited the strength of this meta-analysis. Minimally invasive surgery has evolved rapidly in recent years. As minimally invasive approaches reduce the factors associated with pulmonary complications (*e.g.*, blood loss, pain and inflammation), minimally invasive esophagectomy would be particularly beneficial with respect to pulmonary complications. In the included studies, three studies performed thoracoscopy-assisted surgery^[22-24], and two studies performed subtotal esophagectomy^[23,26]; therefore, the different procedures adopted in these studies would also have some effect on the results.

The results for perioperatively administered neutrophil elastase inhibitor are encouraging. All the trials included were conducted in Eastern populations, and genomic factors may have influenced the results^[36,37]. Further trials are required in other areas to determine whether these results can be extrapolated to all populations.

In summary, neutrophil elastase inhibitor administration is beneficial in patients undergoing esophagectomy, especially in terms of the duration of mechanical ventilation, pulmonary function, pulmonary complications and SIRS state. Although many studies have reported that it also plays an active role in ICU stay and hospital stay, there is currently insufficient evidence for these effects, and more high-quality, large sample, multi-center and randomized controlled trials are needed.

COMMENTS

Background

Esophageal carcinoma is the sixth leading cause of cancer-related deaths worldwide. Patients undergoing radical esophagectomy suffer excess surgical stress, which mainly causes pulmonary complications. Sivelestat sodium hydrate is recommended for the treatment of acute lung injury, and is considered effective in patients with esophageal carcinoma undergoing esophagectomy. However, this needs to be systematically evaluated.

Research frontiers

This meta-analysis was performed to evaluate the benefit and safety of sivelestat administration in patients undergoing esophagectomy. The outcome measures included mechanical ventilation, pulmonary complications, SIRS, ICU

stay, postoperative hospital stay and other complications.

Innovations and breakthroughs

This meta-analysis revealed that sivelestat is beneficial in patients undergoing esophagectomy, especially in terms of the duration of mechanical ventilation and the incidence of pulmonary complications. It may also play an active role on ICU stay, hospital stay, oxygenation and blood cytokine levels. However, there is currently insufficient evidence for these effects.

Applications

The current analysis shows that sivelestat sodium hydrate may achieve better treatment outcomes in patients with esophageal carcinoma undergoing esophagectomy. Sivelestat reduced the duration of mechanical ventilation support and the incidence of pulmonary complications. In addition, side effects did not appear to be a concern.

Terminology

Radical esophagectomy, which mainly consists of video-assisted thoracoscopic esophagectomy, cervical esophagogastronomy and two- or three-field lymph node dissection, is one of the most invasive surgical techniques performed in the gastrointestinal system.

Peer-review

This is a nicely written manuscript with a thoroughly performed review and meta-analysis on the use of sivelestat perioperatively for esophagectomy, and the outcomes and analyses were really conducive.

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Pyogenic liver abscess caused by *Fusobacterium* in a 21-year-old immunocompetent male

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Abstract

A 21-year-old male with no significant past medical history, presented with right upper quadrant (RUQ) abdominal pain along with fevers and chills. Lab work revealed leukocytosis, anemia, and slightly elevated alkaline phosphatase. Viral serology for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus were negative and he was immunocompetent.

Computed tomography imaging revealed hepatic abscesses, the largest measuring 9.5 cm. Empiric antibiotics were started and percutaneous drains were placed in the abscesses. Anaerobic cultures from the abscesses grew *Fusobacterium nucleatum*. This is a gram negative anaerobic bacteria; a normal flora of the oral cavity. *Fusobacterium* is most commonly seen in Lemiere's disease, which is translocation of oral bacteria to the internal jugular vein causing a thrombophlebitis and subsequent spread of abscesses. Our patient did not have Lemiere's, and is the first case described of *Fusobacterium* pyogenic liver abscess in a young immunocompetent male with good oral hygiene. This case was complicated by sepsis, empyema, and subsequent abscesses located outside the liver. These abscesses have the propensity to flare abruptly and can be fatal. This case not only illustrates *Fusobacterium* as a rare entity for pyogenic liver abscess, but also the need for urgent diagnosis and treatment. It is incumbent on physicians to diagnose and drain any suspicious hepatic lesions. While uncommon, such infections may develop without any overt source and can progress rapidly. Prompt drainage with antibiotic therapy remains the cornerstone of therapy.

Key words: Hepatic abscess; Pyogenic; *Fusobacterium*; Liver; Immunocompetent

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Core tip: Pyogenic liver abscesses have the propensity to cause devastating effects; immediate drainage and antibiotics are the mainstay of treatment. Herein we report the first case of a pyogenic liver abscess from *Fusobacterium* in a young, otherwise healthy immunocompetent individual.

Ahmed Z, Bansal SK, Dhillon S. Pyogenic liver abscess caused by *Fusobacterium* in a 21-year-old immunocompetent male.

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INTRODUCTION

Pyogenic liver abscesses (PLA) are uncommon entities which require urgent diagnosis and treatment. If untreated they carry a high mortality. Hepatic abscess due to *Fusobacterium* (gram negative anaerobic bacteria) is rare, but when reported they are associated with Lemierre's disease, an infectious thrombophlebitis of the internal jugular vein. Such infections are typically seen in the setting of instrumentation, dental work, and incidental oral flora translocation. Herein, we present the first case of *de novo* hepatic abscesses due to *Fusobacterium* in an otherwise healthy 20-year-old Caucasian male with no identifiable source of infection. His course was further complicated by development of multiple intra-abdominal abscesses with subsequent direct seeding into the right pleural cavity causing empyema.

CASE REPORT

A 20-year-old Caucasian male was admitted with two weeks of progressive right upper quadrant abdominal pain, approximately 10 pound weight loss, fevers/chills, fatigue, and diarrhea. His social history was not significant for any IVDA, high risk sexual behavior, recent travel, exposure to contaminated water, or pet exposure. He was a college student and played baseball on the school team. On presentation his vital signs were stable and was afebrile. Examination revealed mild RUQ tenderness, but was otherwise unremarkable. His laboratory work revealed: leukocytosis with wbc count of 16.3 k/uL and anemia with Hgb of 7.6 gm/dL. Liver function tests revealed normal: AST (29 U/L)/ALT (44 U/L), total bilirubin (0.7 mg/dL); and mild elevation of alkaline phosphatase (158 U/L). Serologic workup was negative for human immunodeficiency virus, hepatitis B virus and hepatitis C virus. Computed tomography (CT) imaging of abdomen/pelvis revealed multiple hepatic abscesses, the largest in the right lobe measuring 8.9 cm × 9.4 cm × 9.5 cm (Figure 1A). Aerobic and anaerobic blood cultures were collected and empiric antibiotic treatment was initiated with Vancomycin and Piperacillin/Tazobactam. Two percutaneous drains were placed in the largest hepatic abscesses. The hepatic abscesses' anaerobic culture grew heavy *Fusobacterium nucleatum* (*F. nucleatum*). The patient denied any neck pain or recent dental infection, but did report recent routine dental cleaning. Neck imaging revealed patent jugular veins without tenderness or palpable cords. An ERCP was done to rule out a biliary source, and revealed normal hepatic, biliary, and

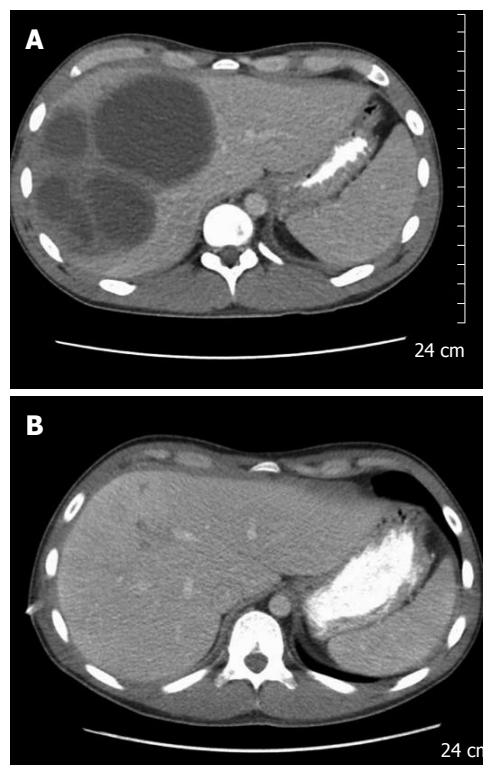


Figure 1 Abdominal computed tomography. A: Hepatic abscess, prior to drainage and antibiotics. Largest hepatic abscess seen measuring 8.9 cm × 9.4 cm × 9.5 cm; B: Resolution of hepatic abscesses after drainage and antibiotics.

pancreatic ducts.

One week after initiating treatment the patient developed respiratory distress and was found to have a large right sided multiloculated pleural effusion for which a chest tube was placed and pleural fluid analysis revealed empyema, likely due to direct extension of the pyogenic liver abscesses. The chest tube did not resolve the pleural effusion and the patient subsequently developed a trapped lung. He underwent evacuation of the pleural fluid and partial decortication with a VATS procedure. He developed 2 more abdominal abscesses, and one pelvic abscess, all required percutaneous drain drainage with resolution. A 2D trans-thoracic echocardiogram was unremarkable, apart from a mild pericardial effusion. The patient was placed on IV Ertapenem for 8 wk and discharged home in stable condition. Repeat imaging during out-patient follow-up showed complete resolution of his abscesses after 9 wk of treatment (Figure 1B).

DISCUSSION

Liver

The two most common types of liver abscesses are: PLA and amebic abscess. PLA is defined as an encapsulated mass containing purulent material^[1-3]. Incidence varies with geographical location; PLA are more common in developed countries whereas amebic abscess are more common in developing countries.

For PLA the reported incidence in the United States is 3.6 out of 100000 people^[4,5]. The right hepatic lobe is the most common site of involvement, due to its larger size and having greater blood supply when compared to the left and caudate hepatic lobes^[6]. PLA are more commonly polymicrobial rather than monomicrobial. Meddings *et al*^[5], evaluated close to 18000 patients with PLA in the United States; and found the most commonly recorded bacterial infections were: *Streptococcus* species (29.5%) and *Escherichia coli* (18.1%). Of note, Taiwan had the highest reported incidence at 17.6 out to 100000 people, with *Klebsiella* being the most prominent bacterial organism being found. Anaerobic species are also not uncommon in such patients, with *Bacteroides* being most commonly isolated. Anaerobes are probably under-reported because of difficulty associated with culturing them. There are only a few case reports, which report *Fusobacterium* as the causative organism. Of the two large PLA studies done by Meddings *et al*^[5] and Chuang *et al*^[7], none of them were reported to have been caused by *Fusobacterium*.

Liver abscess can occur from three mechanisms: (1) dissemination *via* the portal venous system is most common cause of pyogenic liver abscess. Intra-abdominal infections in portal bed region organs, result in septic thrombophlebitis causing micro-abscess formation in the liver. These micro-abscesses later coalesce to form larger single or multiple abscesses; (2) biliary tract disease such as biliary duct stone, stricture or malignancy, results in direct extension of infection through biliary channels to liver; and (3) hematogenous spread from systemic bacteremia can result from endocarditis, septic thrombophlebitis (Lemierre's syndrome) or periodontal infections^[8].

Notable risk factors for PLA include: diabetes mellitus, liver transplantation, intra-abdominal malignancy, biliary tract procedures, and immune system suppression^[9]. Several recent studies from Taiwan mention an association between *Klebsiella pneumoniae* and colorectal cancer. Their studies show patients diagnosed with PLA had a fourfold increase in gastrointestinal malignancy, of which colorectal cancer was the most common^[10-15].

Treatment of PLA includes prompt initiation of intravenous antibiotics and drainage of purulent material. The preferred method is *via* percutaneous drainage, with needle aspiration being an inferior choice due to requirement of repeated aspirations. Surgical intervention is recommended for: abscesses > 5 cm, gas forming organism, or biliary fistulization. Empiric broad spectrum intravenous antibiotics should include one of the following: ampicillin/sulbactam, ceftriaxone plus metronidazole, or meropenem. Definitive therapy is based on speciation of isolated organism. Recommended duration of antibiotics varies, usually ranging from 2-6 wk; depending on clinical improvement and resolution of abscess seen on CT

imaging^[3].

Fusobacterium

The genus *Fusobacterium* currently includes 13 species and belongs to family bacteroidaceae^[16,17]. Most common species of *Fusobacterium* responsible for human infections are *Fusobacterium necrophorum* (*F. necrophorum*) and *F. nucleatum*. *Fusobacterium* is a nonsporeforming, nonmotile, gram negative small spindle-shaped rod^[17,18]. *F. nucleatum* is part of normal oral flora^[18,19]. *Fusobacterium* species possesses outer membrane proteins like other gram-negative bacteria^[17]. The Lipopolysaccharides in the outer membrane proteins can act as endotoxins and provide other antigenic properties such as adhesion and co-aggregation which helps in invasion of the tissue and contribute towards virulence^[17].

In 1936, Dr. Lemierre was the first to describe a periodontal source leading to septicemia from *Fusobacterium*. *F. necrophorum* is associated with younger healthy population while *nucleatum* is mostly associated with older patients with chronic medical conditions^[20]. *F. nucleatum* had been associated with infections such as tropical skin ulcers, peritonsillar abscesses, pyomyositis, septic arthritis, bacteremia, intrauterine infections, bacterial vaginosis, urinary tract infections, pericarditis, endocarditis, myocarditis, and pulmonary infections^[17,21]. *F. Nucleatum* has also recently been implicated as causative agent in chorioamnionitis and idiopathic preterm labor^[16]. Association of *F. Nucleatum* infections with GI tract malignancies is attributed to the breach in mucosal lining resulting in invasive infections^[18].

Annual incidence of *Fusobacterium* infections is between 0.6-3.5 cases per 1000000 people^[20]. Su *et al*^[22] pointed out that most *Fusobacterium* blood stream infections were from *nucleatum* species. Community acquired *F. nucleatum* infections tend to be more polymicrobial as compared to nosocomial which are almost always monomicrobial^[23,24]. Risk factors for poor prognosis were presence of comorbid conditions such as diabetes mellitus, renal failure, dialysis and malignancy^[18,20]. Nosocomial bacteremia was a significant mortality predictor with rates as high as 84% in one study^[18]. *F. nucleatum* bacteremia without identifiable source is not uncommon. In such cases, a GI tract malignancy is often found^[18].

The *Fusobacteria* species are broadly susceptible to commonly used antibiotics, but reports of increasing resistance to: vancomycin, neomycin, erythromycin, amoxicillin, ampicillin, and phenoxymethylpenicillin; have emerged^[17]. Recently some resistant patterns to quinolones had been identified with *Fusobacteria* isolated from oral flora of dogs and cats^[16]. An accurate identification of *Fusobacterium* species is critical not only for taxonomic reasons but also for appropriate treatment of infection, since the susceptibility of different *Fusobacterium* species to antibiotics varies

widely^[17]. Only 13 cases have been reported of liver abscesses caused by *F. nucleatum*, 12 of which were in immunosuppressed individuals, and 1 mentioned by Kajiya *et al.*^[25] was an elderly immunocompetent individual^[26-29]. Apart from our case, no other case has been reported in an otherwise young and healthy immunocompetent patient.

Pyogenic liver abscess from fusobacterium is a rare diagnosis and behaves clinically like any other anaerobic body cavity infections. It presents with fever, chills and weight loss in most cases. Surprisingly, jaundice and RUQ pain are inconsistent findings^[8]. Course is indolent which could be used as a clinical clue on initial presentation. Necrosis of the tissue with putrid discharge and abscess formation are seen in later stages of the disease. Anemia and weight loss are indicators of long standing chronic infection. The spectrum of infections that can occur with *Fusobacterium* species is broad and carry a high mortality if untreated. Suspicion for anaerobic infections such as *Fusobacterium* should be kept high when a host with risk factors presents with indolent disease course. Also it should be kept in mind that while often seen in immunocompromised individuals, it can present, as in our case, in otherwise healthy individuals. It is postulated the patient got this invasive strain from possible plaques and/or minor oral cavity manipulation during routine dental cleaning. Prompt drainage along with empiric anaerobic coverage is recommended initially. The sequelae of this condition are fatal, prompt diagnosis and treatment with source control being the focus are crucial in preventing mortality.

COMMENTS

Case characteristics

A 21-year-old immunocompetent male with pyogenic liver abscess from *Fusobacterium*.

Clinical diagnosis

Right upper quadrant pain, fevers/chills, and weight loss in an otherwise healthy patient.

Differential diagnosis

Hepatitis, hepatic cancer, biliary obstruction.

Laboratory diagnosis

WBC count of 16.3 k/uL; Hgb of 7.6 gm/dL; liver function tests relatively within normal limits; liver serology negative.

Imaging diagnosis

Computed tomography imaging of abdomen/pelvis revealed multiple hepatic abscesses, the largest in the right lobe measuring 8.9 cm x 9.4 cm x 9.5 cm.

Pathological diagnosis

Culture of aspirates showing: gram negative anaerobe-*Fusobacterium nucleatum* (*F. nucleatum*), otherwise pathology negative.

Treatment

Aspiration and drainage, along with IV antibiotics: Ertapenem.

Related reports

Pyogenic liver abscesses in otherwise healthy individuals is not very common, case reports describe: invasive oral cavity manipulation, biliary translocation, and gastrointestinal migration as causes; none of which were present in this case.

Term explanation

PLA is used to denote pyogenic liver abscess.

Experience and lessons

This case report illustrates importance of recognizing pyogenic liver abscess as a possible entity in otherwise healthy individuals with no predilection, and urgent treatment with: Drainage and antibiotics.

Peer-review

The author presented a young, immunocompetent patient had liver abscesses caused by *F. nucleatum*, a nonsporeforming, nonmotile, gram negative small spindle-shaped rod. *F. nucleatum* is a rare cause of pyogenic liver abscess and high morbidity and mortality will happen if delayed diagnosis and treatment. It is interesting to readers to keep in mind if same scenario happened.

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Complicated fecal microbiota transplantation in a tetraplegic patient with severe *Clostridium difficile* infection

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Abstract

A 65-year-old male suffering from acute spinal cord injury leading to incomplete tetraplegia presented with severe recurrent *Clostridium difficile* (*C. difficile*) infection subsequent to antibiotic treatment for pneumonia. After a history of ineffective antimicrobial therapies, including metronidazole, vancomycin, fidaxomicin, rifaximin and tigecycline, leading to several relapses, the patient underwent colonoscopic fecal microbiota transplantation from his healthy son. Four days subsequent to the procedure, the patient showed a systemic inflammation response syndrome. Without detecting an infectious cause, the patient received antimicrobial treatment, including tigecycline, metronidazole, vancomycin *via* polyethylene glycol and an additional enema for a period of seven days, leading to a prompt recovery and no reported *C. difficile* infection relapse during a 12 wk follow up.

Key words: *Clostridium difficile* infection; Spinal cord injury; Fecal microbiota transplantation; Systemic inflammatory response syndrome

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Core tip: A 65-year-old male suffering from incomplete tetraplegia presented with severe recurrent *Clostridium difficile* (*C. difficile*) infection subsequent to antibiotic treatment of pneumonia. After several antimicrobial therapies with subsequent relapses, the patient underwent colonoscopic fecal microbiota transplantation

from his healthy son. Four days later, the patient showed a systemic inflammation response syndrome. Without detecting an infectious cause, the patient received antimicrobial treatment, including tigecycline, metronidazole, vancomycin *via* polyethylene glycol and an additional enema, for a period of seven days, leading to prompt recovery and no reported *C. difficile* infection relapse during a 12 wk follow up.

Brechmann T, Swol J, Knop-Hammad V, Willert J, Aach M, Cruciger O, Schmiegel W, Schildhauer TA, Hamsen U. Complicated fecal microbiota transplantation in a tetraplegic patient with severe *Clostridium difficile* infection. *World J Gastroenterol* 2015; 21(12): 3736-3740 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3736.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3736>

INTRODUCTION

The recurrence rate of *Clostridium difficile* (*C. difficile*) infections (CDI) after adequate antibiotic treatment remains high and is even more frequent in patients who once relapsed^[1]. Data analysis of CDI in spinal cord injured (SCI) patients is insufficient but recurrence rates seem to be equivalent to the non-SCI population^[2]. A recent systematic review provides evidence that fecal microbiota transfer (FMT) seems to be a useful adjunct in the treatment of CDI relapses^[3]. Out of 536 patients enrolled and treated with FMT, 467 (87%) succeeded in showing no further symptoms. However, success rates vary depending on the site of administration: 81% (stomach); 86% (duodenum and jejunum); 93% (cecum and ascending colon); and 84% for the distal colon. No severe adverse events occurred due to the procedure.

Patients with SCI present with special characteristics in the course of gastrointestinal diseases^[4-6] and therefore the management and treatment is challenging^[7,8]. For example, impaired colonic motility leads to decreased large bowel transport, frequently leading to constipation or megacolon^[9,10]. Furthermore, high level tetraplegic patients (C4 or higher), with more frequent administration of antibiotics due to permanent or intermittent dependency on assisted ventilation following ventilator associated pneumonia, are more susceptible to colonization with multi resistant bacteria or *C. difficile* infections^[11].

Yet, no special protocols for the treatment of CDI in tetraplegic patients have been published, so general regimens are applied. Until now, no FMT procedure in SCI patients has been described in the available literature.

CASE REPORT

A 65-year-old male was admitted to our hospital after ventral spondylodesis of C3 to C5 due to traumatic

Table 1 Antibiotic treatment prior to fecal microbiota transfer procedure

Episode	Antibiotic regimen	Dose	Administration route	Length
1	Vancomycin	4 times/250 mg	PEG	10 d
	Vancomycin	2 times/2 g	Enema	10 d
	Metronidazole	3 times/400 mg	PEG	10 d
2	Fidaxomicin	2 times/200 mg	PEG	10 d
3	Rifaximin	3 times/400 mg	PEG	10 d
4	Tigecycline	1 time/50 mg	<i>iv</i>	10 d
5	Rifaximin	3 times/400 mg	PEG	14 d
6	Tigecycline	1 time/50 mg	<i>iv</i>	7 d
	Vancomycin	2 times/2 g	Enema	7 d
	Rifaximin	3 times/400 mg	PEG	10 d
7	FMT-procedure			

PEG: Polyethylene glycol; FMT: Fecal microbiota transfer.

fracture. However, high level incomplete tetraplegia (American Spinal Injury Association impairment scale C) persisted so that intermittent mechanical ventilation *via* tracheostoma and enteral feeding *via* gastric tube [polyethylene glycol (PEG)] became necessary. After several weeks of intensive care treatment, transfer to a general ward was possible. The patient received antibiotic treatment (co-trimoxazole and imipenem) because of a ventilator-associated pneumonia. As a consequence of this, 10 wk post trauma, he presented with watery diarrhea. An Elisa immunoassay detected *C. difficile* toxin A and B and polymerase chain reaction as a nucleic acid amplification method distinguished *C. difficile* ribotype 027.

Several therapeutic protocols including metronidazole, vancomycin, fidaxomicin, rifaximin and tigecycline in different forms of applications and combinations were administered (Table 1). Treatment was initiated with vancomycin *via* PEG 250 mg 4 times a day and *via* clyster. Metronidazole was added *via* PEG 400 mg 3 times a day. No vancomycin tapering was done. Fidaxomicin was given using 200 mg *via* PEG two times a day for 10 d. Due to the next relapse, the patient was treated with rifaximin 400 mg three times a day *via* PEG for 2 wk. After the next relapse, he was treated with tigecycline intravenously for 10 d combined with vancomycin 2 g *via* clyster.

None of the above succeeded in resolving the symptoms. A total of six relapses occurred, four of which were confirmed by toxin testing in a stool sample. Two episodes without microbiological proof were highly probable by clinical evaluation. Two of the recurrences matched the criteria of severe CDI.

After the sixth treatment failure and renewed cultural proof of CDI, we decided to transfer fecal microbiota. The son, as a healthy first degree relative, gave informed consent for FMT. He reported no antibiotic treatment in the last three months prior to the procedure. Additional testing for infectious diseases (anti-hepatitis A virus, anti-hepatitis B core, hepatitis B surface antigen, anti-hepatitis C virus,

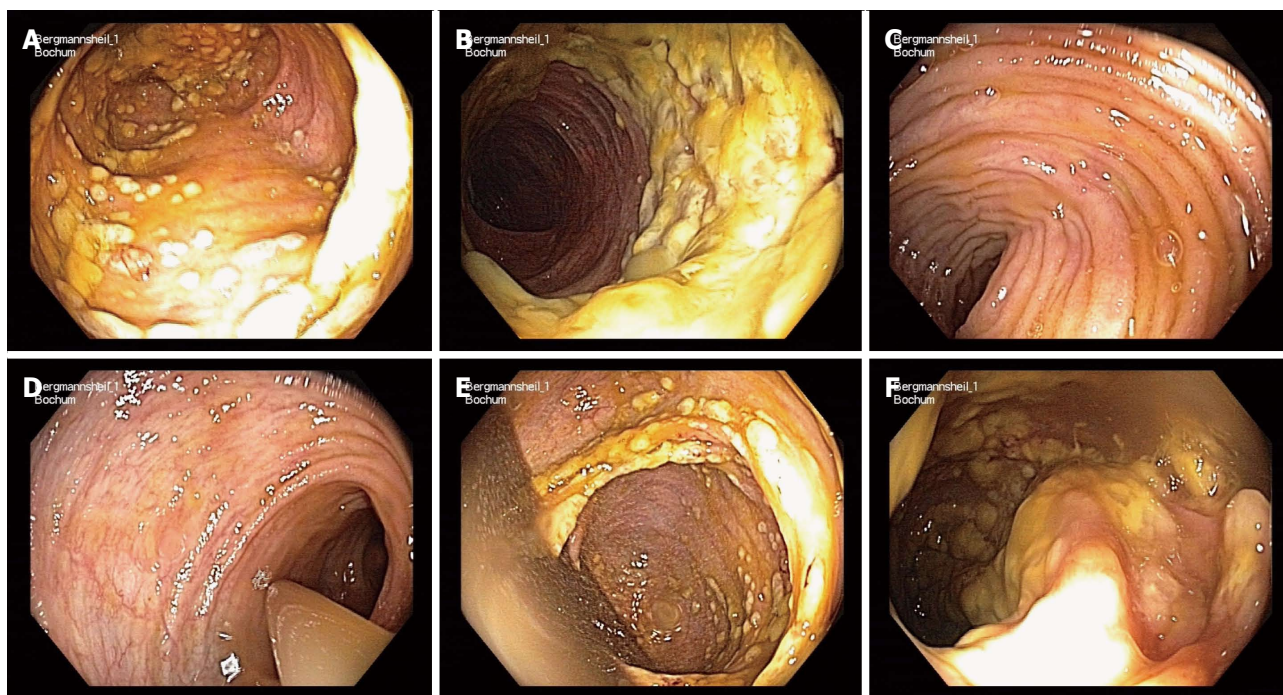


Figure 1 Endoscopic appearance during colonoscopic fecal microbiota transfer procedure. A, B: Severe pseudomembranous colitis in the colon after antibiotic treatment and bowel lavage; C: Terminal ileum without endoscopic changes; D: Instillation of fecal transplant; E, F: Fecal transplant in pseudomembranous colitis areas.

human immunodeficiency virus, cytomegalovirus, Epstein-Barr virus, Treponema pallidum) in the serum and *C. difficile* toxins A and B, campylobacter spp., Shigella, salmonella, Yersinia, pathogenic Escherichia coli, Adeno-, Rota- and Norovirus, parasites, Cryptosporidium/Microsporidium in three independent fecal samples were negative.

In order to prepare the patient for the FMT procedure, we omitted antibiotic treatment over a period of one week and bowel lavage and enema was performed on the 7th day. Fresh donor stool was immediately transferred to the laboratory in an airtight container. A portion of 160 g was weighed and homogenized with isotonic sodium chloride in a sterile flask. Afterwards, the homogenate was filtered repeatedly through a sterile gauze pad to remove as much particulate matter as possible^[12]. The finished stool slurry was immediately transferred to the endoscopy unit and administered into the terminal ileum and colon during colonoscopy. The endoscopic findings showed severe pseudomembranous colitis with destruction of large areas of the colonic surface (see Figure 1).

Four days subsequent to the FMT, the patient experienced fever as part of a severe systemic inflammatory response syndrome with shock. Stool consistency was pulpy but not watery as seen before. Due to respiratory failure and vasoplegia which required immediate mechanical ventilation and high-dose vasopressor therapy, the patient was transferred from the intermediate care to the intensive care unit. Extensive work-up did not reveal any infectious cause. Whole body CT scan did not show a focus despite the

persistent colitis, especially no signs of pneumonia, empyema or soft tissue infection. Microbiological testing did not detect *C. difficile* in the stool sample and blood and urine cultures did not detect any bacteria or mycosis. There was no sign of catheter-associated blood stream infection. Echocardiographic examination did not show endocarditis.

We initiated calculated intravenous antibiotic therapy, including tigecycline and metronidazole as well as vancomycin *via* PEG and an enema, for a period of seven days. The respiratory and cardiovascular situation improved shortly after initiation of the treatment. In the following weeks, the patient did not receive any further antibiotic therapy. Weaning from ventilation was successful within the next weeks, enabling transfer of the patient to a specialized rehabilitation clinic. No recurrence of watery diarrhea or CDI-like symptoms occurred in the following twelve weeks subsequent to the FMT procedure.

DISCUSSION

Only very few adverse events associated with FMT have been described in the available literature so far, focussing on general population^[13]. In more than 200 fecal transplantation procedures at the Academic Medical Centre of Amsterdam, The Netherlands, and more than 3000 at the Centre for Digestive Diseases in Sydney, Australia, no serious adverse event have been reported. Most patients treated with FMT experienced diarrhea on the day of the procedure and only a minority of the patients reported belching, abdominal cramping or constipation. These observations coincide

with published case reports and series of FMT for CDI^[14,15]; adverse events were reported for only 3 of 317 patients (upper gastrointestinal tract bleeding, peritonitis or enteritis). In another case report, nasoduodenal FMT for Crohn's disease resulted in transient adverse effects, such as fever and abdominal tenderness in three out of four patients. However, the symptoms were temporary and ceased spontaneously in the following two days post FMT^[16]. A case report by Borody *et al.*^[17], in which FMT was administered during colonoscopy, does not describe any side effects due to the procedure. Substantially, long-term follow up studies state FMT as a safe and feasible procedure without major adverse events^[18].

Due to impaired bowel function, among other characteristics, patients with spinal cord injury are a challenging population. In particular, CDI in SCI-patients seems to take a more severe clinical course^[19]. Furthermore, bowel preparation as the initial step of FMT is less effective^[8], which may lead to deterioration of the FMT outcome.

This case emphasizes that FMT is feasible and effective in the treatment of CDI in tetraplegic patients; however, possible adverse and even severe adverse events have to be taken into consideration. In a population of ninety-nine immunosuppressed patients, severe adverse events occurred in 15% of the patients, including one procedure-associated and one non procedure-associated death^[20]. Astonishingly, infection has not been the leading problem. Although our patient showed symptoms of systemic inflammation response syndrome, an infectious cause was not identified during the extensive diagnostic work-up. We hypothesize that some bacteria, possibly *C. difficile*, might have passed the severely destroyed mucosal layer during colonoscopy of the insufficiently prepared large bowel. The assumed transient bacteremia may be avoidable by performing FMT using a nasojejunal tube.

Although the cause remained uncertain, the treatment for septic CDI using metronidazole, vancomycin and tigecycline was initiated. With the absence of diarrhea 12 wk after the FMT procedure, the enterocolitis is considered to be in remission, emphasizing that antibiotic treatment after FMT did not lead to a relapse of CDI in the sense of the international definition^[21,22].

To our knowledge, this is the first case reporting successful fecal microbiota transfer in a SCI-patient with recurrent CDI. Severe adverse events, although rarely described in literature, have to be considered subsequent to FMT, especially in patients with further impairments of bowel function. In cases of tetraplegic patients, oral administration of the stool suspension might be more feasible than through a colonoscope due to the impaired bowel cleansing prior to the procedure and therefore a higher microbial load. However, antibiotic treatment after FMT does not necessarily deteriorate the outcome of fecal microbiota transplantation.

COMMENTS

Case characteristics

A patient with acute spinal cord injury leading to incomplete tetraplegia developed recurrent diarrhea after antibiotic treatment of ventilator associated pneumonia.

Clinical diagnosis

Relapsing *Clostridium difficile* (*C. difficile*) infection (CDI) was likely.

Differential diagnosis

After several regimens to treat CDI, fecal microbiota transplantation (FMT) was planned and undertaken.

Laboratory diagnosis

An ELISA detected *C. difficile* toxins A and B, polymerase chain reaction proved *C. difficile* ribotype 027.

Imaging diagnosis

Colonoscopy during FMT showed pseudomembranous colitis, a computed tomography scan four days later revealed severe colitis; all other examinations did not show pathological signs.

Treatment

Relapsing CDI was treated by FMT, which four days later resulted in a systemic inflammatory response syndrome (SIRS); subsequent treatment with tigecycline, rifaximin and vancomycin led to prompt complete recovery.

Related reports

There is an increasing interest in the literature in FMT but descriptions of complications are scarce. Furthermore, several special groups of patients have been treated with FMT but no tetraplegic patient with functional abnormalities of the gastrointestinal and colonic tract has been described so far.

Experiences and lessons

The authors report the first case of FMT in a tetraplegic patient and describe a possible, procedure related serious adverse event.

Peer-review

This is an interesting case report of CDD treated with FMT in a patient with acute spinal cord injury who had been previously treated with antibiotics. The paper is well written and the case is interesting and potentially useful for readers.

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Fulminant phlegmonitis of the esophagus, stomach, and duodenum due to *Bacillus thuringiensis*

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Author contributions: Matsumoto H participated in data acquisition and drafted the manuscript; Ogura H contributed to the critical appraisal of the manuscript; Seki M made critical contributions to the analysis of data and the drafting of the manuscript; Ohnishi M contributed to the drafting of the manuscript; and Shimazu T supervised the manuscript.

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phlegmonous gastritis in conditions of immunodeficiency.

Key words: Phlegmonous gastritis; Esophagus; Duodenum; *Bacillus thuringiensis*; Immunocompromised state

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Core tip: This is the first reported case of *Bacillus thuringiensis* as the suspected causative agent of rapidly progressive and fatal phlegmonitis of the esophagus, stomach, and duodenum in patient in an immunocompromised state. Even low-virulence bacilli may be a causative pathogen of gastrointestinal phlegmonitis in patients in an immunocompromised state.

Matsumoto H, Ogura H, Seki M, Ohnishi M, Shimazu T. Fulminant phlegmonitis of the esophagus, stomach, and duodenum due to *Bacillus thuringiensis*. *World J Gastroenterol* 2015; 21(12): 3741-3745 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3741.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3741>

INTRODUCTION

Phlegmonous gastritis is a rare acute and occasionally fatal bacterial infection of the gastric wall. Although a number of different bacterial organisms have been implicated as etiologic agents, *Bacillus thuringiensis* has not been reported as such an agent. We report a case of fatal phlegmonous gastritis with *Bacillus thuringiensis* suspected as the pathogen of intestinal infection. To the best of our knowledge, this is the first reported case of this type.

CASE REPORT

A 74-year-old man experiencing acute epigastric

Abstract

We report a case of phlegmonitis of the esophagus, stomach, and duodenum in patient in an immunocompromised state. Culture of gastric juice and blood yielded *Bacillus thuringiensis*. This case showed that even low-virulence bacilli can cause lethal gastrointestinal

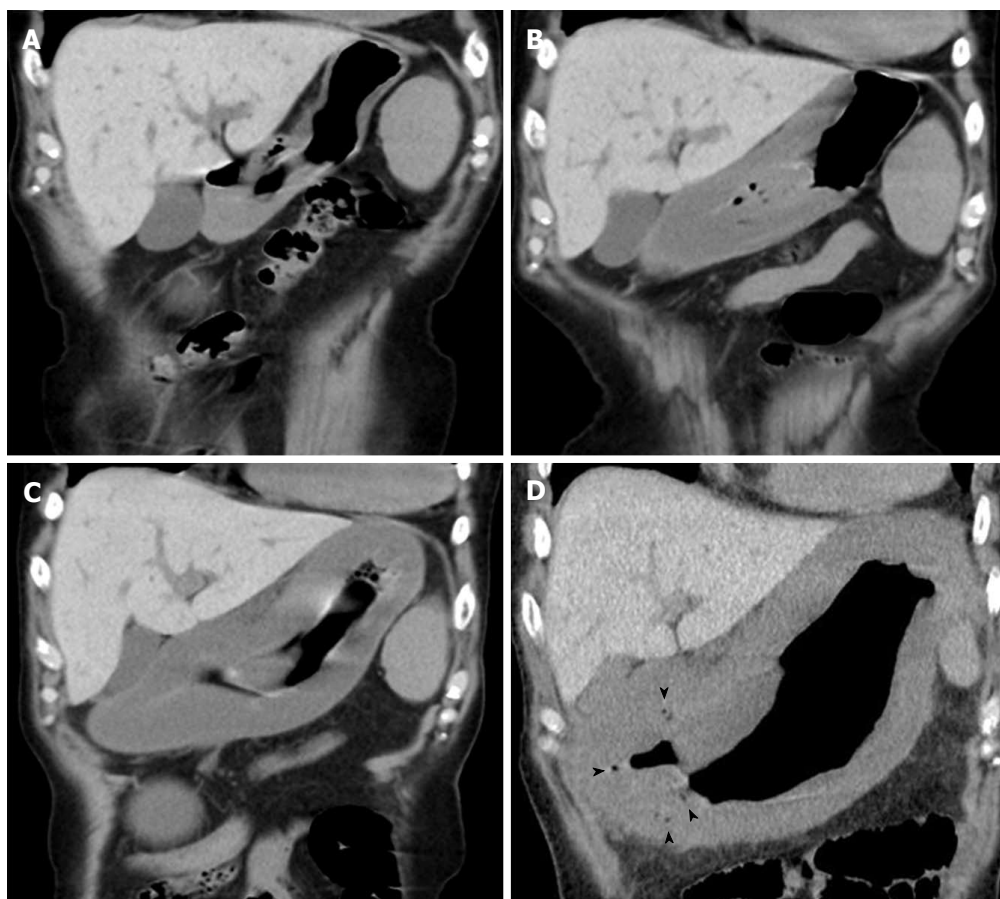


Figure 1 Abdominal computed tomography images show rapid progression of gastric wall hypertrophy. Twenty days before onset (A) and at 4 h (B), 6 h (C), and 10 h (D) after onset. Arrowheads in (D) indicate intramural gas.

pain and nausea after a midnight meal was rushed to an emergency hospital 1 h after onset. He had past histories of myelofibrosis and stage III multiple myeloma^[1]. He had neutropenia as a result of myelosuppressive chemotherapy, and on admission to hospital, his white blood count was 800 cells/mm³ with an absolute neutrophil count of 300 cells/mm³. Compared with a screening computed tomography (CT) scan performed 20 d before onset (Figure 1A), abdominal CT performed 4 h after onset revealed gastric wall hypertrophy (Figure 1B). Abdominal CT performed 6 h after onset showed continued significant spread of the wall hypertrophy to the total stomach (Figure 1C), and upper gastrointestinal endoscopy revealed ischemic change over the entire gastric mucosa. He was transferred to our critical care center 9 h after onset. On arrival, his temperature was 36.7° C, blood pressure was 98/69 mmHg, pulse rate was 113/min, and respiration rate was 36/min, indicating a shock state and systemic inflammatory response syndrome. Physical examination found local muscular guarding in the epigastric area. His blood chemistry data showed kidney and liver dysfunction, with a creatinine level of 2.6 mg/dL, alanine aminotransferase level of 77 IU/L, and aspartate aminotransferase level of 76 IU/L. Arterial blood gas analysis revealed severe

acidosis, with a pH of 7.14 and blood lactate level of 117 mg/dL. Abdominal CT performed at 10 h after onset showed progression of wall hypertrophy to the lower esophagus and total stomach and duodenum and the presence of intramural gas (Figure 1D, arrowheads). Contrast enhancement of the gastric wall was very poor. Repeat upper gastrointestinal endoscopy showed extensive ischemic change to the mucosa and necrosis extending from the lower esophagus to the stomach and duodenal bulb (Figure 2). A diagnosis of acute phlegmonitis of the esophagus, total stomach, and duodenum was made based on the CT images. Gram staining of the patient's gastric juice revealed Gram-positive cocci and Gram-negative rods, and Gram-positive rods were also highlighted (Figure 3). We isolated *Bacillus* species in both blood and gastric juice cultures and genetically confirmed them to be *Bacillus thuringiensis* by sequencing of PCR amplified products of the *groEL* region and by detection of Bt toxins. Therefore, we diagnosed the patient as having acute phlegmonitis of the esophagus, stomach, and duodenum due to *Bacillus thuringiensis* (*B. thuringiensis*). MICs determined with an automated system (MicroScan WalkAway; Siemens, Munich, Germany) included gentamicin, < 2 µg/mL (susceptible); cefazolin, <

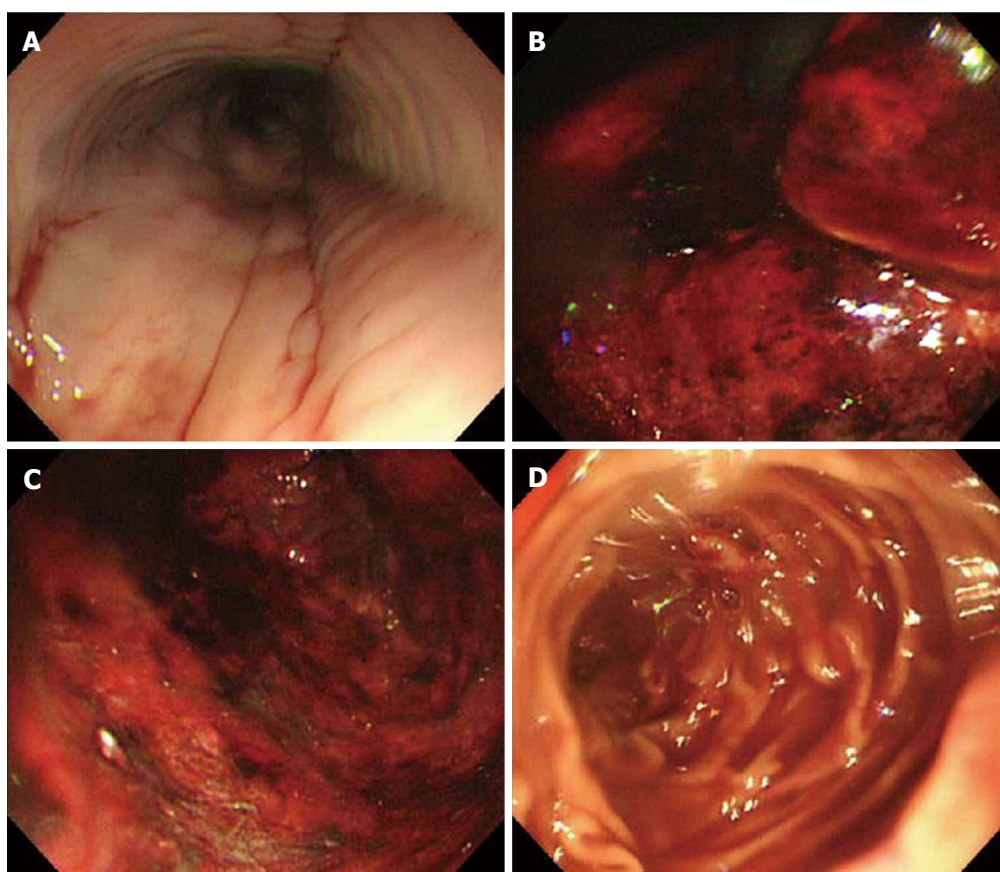


Figure 2 Endoscopy images show extensive mucosal ischemic change and extension of the necrosis. A: Lower esophagus; B: Cardia; C: Gastric corpus; D: Duodenum.

4 $\mu\text{g/mL}$ (susceptible); ciprofloxacin, < 0.5 $\mu\text{g/mL}$ (susceptible); and imipenem, < 1 $\mu\text{g/mL}$ (susceptible).

Despite mechanical ventilation and treatment with meropenem (1 g \times 3/d intravenously) and fluid and vasopressor therapy, the patient rapidly deteriorated due to septic disseminated intravascular coagulation and multiple organ failure. He died 14 h after arrival (24 h after disease onset).

DISCUSSION

Phlegmonous gastritis is caused by bacterial infection invading the gastric wall. Phlegmonitis of the stomach was initially recognized by Cruveilhier in the early 18th century^[2]. Although the pathogenesis of acute phlegmonous gastritis is unclear, several mechanisms are implicated, such as direct invasion through areas of injury and by hematogenous or lymphatic spread. Immunosuppression, alcoholism, achlorhydria, and infection are also reported as predisposing factors^[3]. We considered that myelofibrosis, multiple myeloma, and neutropenia resulting from chemotherapy might have been involved in disease development as predisposing factors for our patient's immunocompromised status.

Phlegmonous gastritis may involve either a portion of the stomach (localized form) or the entire stomach (diffuse form). Phlegmonous involvement

of the esophagus or duodenum beyond the stomach is extremely rare. To our knowledge, only 2 cases of phlegmonous involvement of segments of both the esophagus and duodenum beyond the stomach have been reported^[4,5].

Patients usually present with an acute abdomen and septicemia^[6], and rebound tenderness or muscle guarding can be observed in advanced cases^[7]. Other symptoms include nausea, vomiting, and prostration. CT findings also provide supporting evidence for the diagnosis^[8]. In the present patient, we could clearly detect for the first time, to our knowledge, the time-dependent changes of the spread of fulminant phlegmonitis on screening CT scans. Thus, CT was useful in the early diagnosis of this patient's phlegmonous gastritis.

Submucosal involvement is the most prominent histopathologic finding in phlegmonous gastritis. Snare biopsy specimens are generally more useful for diagnosis because the submucosa is included^[9], but if transmural purulent infection is present in advanced cases, diagnosis may be made by culture of gastric juice, other organs, and blood, as in our patient^[10].

Although primarily caused by β -hemolytic group A *Streptococcus*, phlegmonous gastritis can be caused by many other bacterial organisms^[11]. *B. thuringiensis*, a naturally occurring organism commonly found in soil,

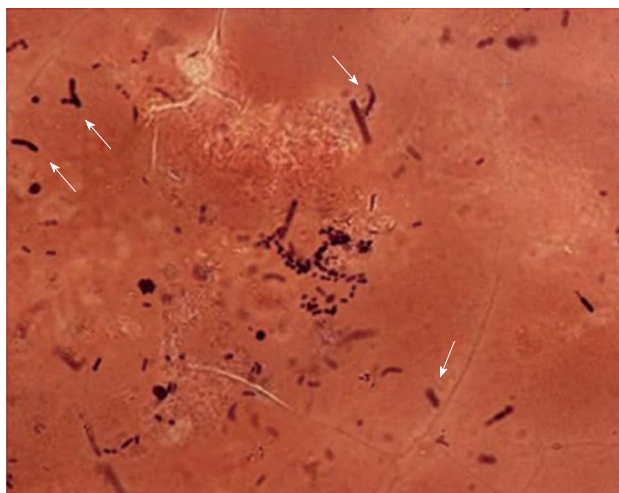


Figure 3 Gram staining of gastric juice from the patient (original magnification $\times 1000$). Arrows indicate *Bacillus thuringiensis*.

has been used for almost half a century as a specific pesticide against various insects^[12]. To our knowledge, there are no other published reports of intestinal infection by *B. thuringiensis*.

Overall, the reported mortality rate for patients with phlegmonous gastritis with medically treated localized disease is 17%, whereas that for diffuse disease is 60%^[6]. Treatment resistance and rapid disease progression were reported to be associated with high mortality^[7]. The factors common to all of the survivors were early recognition and the prompt application of antibiotic treatment or surgery^[13].

In summary, in a patient with rapidly progressive and fatal phlegmonitis spreading to the esophagus, total stomach, and duodenum, *B. thuringiensis* was identified genetically from cultures of gastric juice and blood and was considered the causative pathogen. To our knowledge, this is the first reported case of *B. thuringiensis* relevant to intestinal infection. Even low-virulence bacilli may be a causative pathogen of gastrointestinal phlegmonitis in patients in an immunocompromised state.

COMMENTS

Case characteristics

A 74-year-old man with a history of myelofibrosis and stage III multiple myeloma presented with acute epigastric pain and nausea.

Clinical diagnosis

Local muscular guarding in the epigastric area.

Differential diagnosis

Gastric perforation.

Laboratory diagnosis

White blood count was 800 cells/mm³ with an absolute neutrophil count of 300 cells/mm³, blood chemistry data showed kidney and liver dysfunction, with a creatinine level of 2.6 mg/dL, alanine aminotransferase level of 77 IU/L, and aspartate aminotransferase level of 76 IU/L, and arterial blood gas analysis revealed severe acidosis, with a pH of 7.14 and blood lactate level of 117 mg/dL.

Imaging diagnosis

Abdominal computed tomography showed progression of gastric wall

hypertrophy to the lower esophagus and total stomach and duodenum and the presence of intramural gas.

Pathological diagnosis

The authors isolated *Bacillus* species in both blood and gastric juice cultures and confirmed *Bacillus thuringiensis* (*B. thuringiensis*) by sequencing of PCR amplified products of the *groEL* region and by detection of *B. thuringiensis* toxins.

Treatment

The patient was treated with mechanical ventilation and meropenem (1 g \times 3/d intravenously) and fluid and vasopressor therapy.

Related reports

Phlegmonous gastritis is caused by bacterial infection invading the gastric wall. Many bacterial organisms such as *Staphylococcus* spp. have been implicated as etiologic agents. Immunosuppression, alcoholism, achlorhydria, and infection are reported as predisposing factors. Phlegmonous involvement of the esophagus or duodenum beyond the stomach is extremely rare.

Term explanation

GroEL, one member of the heat shock protein family, which is highly conserved and essential to *Bacillus*, and its gene are used for detection and differentiation of cells belonging to the *Bacillus* spp. group. *B. thuringiensis* toxins are proteins produced by a bacterium that is lethal to insects.

Experiences and lessons

The authors demonstrated for the first time data relevant to intestinal infection by *B. thuringiensis*. The data suggest that *B. thuringiensis* may have been one of the causes of the lethal pathological condition that occurred in a patient in an immunocompromised state.

Peer-review

B. thuringiensis was a relatively mild pathogen in the blood of this patient. Based on the previous history of this individual, the immunosuppressive status of the patient is a good reason to explain the fatal outcome caused by a relatively mild pathogen.

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Cetuximab and panitumumab in a patient with colon cancer and concomitant chronic skin disease: A potential beneficial effect on psoriasis vulgaris

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Abstract

Monoclonal antibodies against epidermal growth factor receptor (EGFR) are used in the treatment of advanced colorectal cancer. However, these agents can induce severe dermatological side effects that discourage their administration in patients with chronic dermatological disease. EGFR plays a key role in normal skin development and immunological function, and is expressed in various tissues and organs, although contrarily, it is overexpressed in psoriasis-related skin lesions. Thus, discussion is ongoing regarding the putative pathological role and therapeutic potential of this protein. We herein report on a patient with advanced colon cancer and concomitant long-standing psoriasis vulgaris who received anti-EGFR antibody monotherapy as a third-line treatment for metastatic disease. One week after the initiation of treatment, the patient's skin lesions dramatically subsided and the improvement was sustained during therapy. Based on this case, we propose that anti-EGFR antibody therapy is not necessarily contraindicated in patients with psoriasis vulgaris. Moreover, the findings reaffirmed that EGFR is an important molecule in the pathology of psoriasis.

Key words: Psoriasis; Cetuximab; Panitumumab; Epidermal growth factor receptor; Colorectal cancer

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Core tip: Anti-epidermal growth factor receptor (EGFR) antibodies are effective in treating advanced colorectal cancer. However, anti-EGFR antibodies are not generally used in patients with concomitant chronic skin disease due to dermatological toxicities. In this case report, we present a patient with psoriasis vulgaris whose symptoms lessened during treatment with anti-EGFR antibody monotherapy for metastatic colon cancer.

Based on this result, we consider that patients with concomitant skin disease should still be considered for anti-EGFR antibody therapy.

Okamoto K, Maeda H, Shiga T, Shiga M, Dabanaka K, Hanazaki K, Kobayashi M. Cetuximab and panitumumab in a patient with colon cancer and concomitant chronic skin disease: A potential beneficial effect on psoriasis vulgaris. *World J Gastroenterol* 2015; 21(12): 3746-3749 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3746.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3746>

INTRODUCTION

Psoriasis vulgaris is a relatively common chronic inflammatory skin disease, which prevalence is ranging from 2.2% to 2.6% in the United States and 0.4% in Asia^[1]. Psoriasis is characterized pathognostically by well-demarcated and slightly elevated red plaques with silver or white scale, reflecting the typically increased vascularity and keratinocyte hyper-proliferation^[2]. Local therapies are prescribed for mild forms of the disease, while phototherapy and systemic therapies such as immunosuppressive drugs and anti-tumor necrosis factor (TNF) antibody are chosen for moderate-to-severe psoriasis, with recent studies highlighting the immune system, and particularly the TNF/NF- κ B/interleukin (IL)-23-Th17 axis, as a pathogenic hub of psoriasis vulgaris^[1]. These studies thus significantly and directly contributed to the development of a new treatment strategy for psoriasis.

Elevated serum levels of epidermal growth factor (EGF) and excessive expression of EGF receptor (EGFR) in affected skin have also been reported in patients with psoriasis vulgaris^[3,4], suggesting pathological keratinocyte proliferation through the EGFR signaling pathway. However, to our knowledge, studies on EGF signaling biology and the potential therapeutic effect of anti-EGFR antibody are scarce in the psoriasis literature^[5-8]. In this case report, we describe a patient with advanced colon cancer and concomitant psoriasis vulgaris, who received anti-EGFR antibody monotherapy using cetuximab and panitumumab.

CASE REPORT

A 55-year-old male patient was referred to our department for the treatment of colon cancer. He was diagnosed with psoriasis vulgaris at 27 years of age, and since then has received topical therapy with corticosteroids and activated vitamin D3 analogue, narrow-band ultraviolet B (NB-UVB) phototherapy, immunosuppressive drug therapy, and oral etretinate, resulting in partial and temporary relief of the symptoms. At the age of 54 years, he received anti-TNF antibody therapy and subsequently, showed

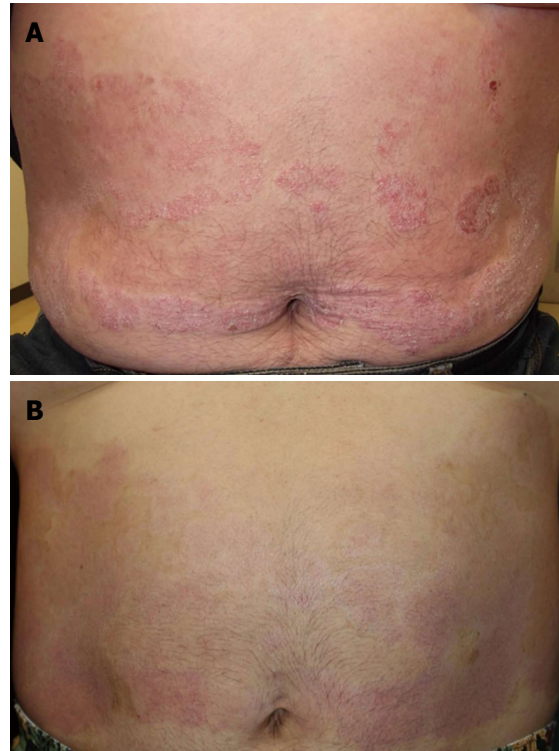


Figure 1 Improvement in psoriasis vulgaris after anti-epidermal growth factor receptor antibody monotherapy. A: Clearly demarcated plaques with silver scales are evident before initiation of cetuximab; B: After initiation of cetuximab, psoriasis skin lesions remarkably improved, and only erythema was observed.

marked improvement in his skin lesions. However, during the treatment, sigmoid colon cancer with multiple liver and lung metastases was identified.

On presentation to our clinic, he underwent laparoscope-assisted sigmoidectomy for the prevention of colonic obstruction and drug therapy with CapeOX (capecitabine, 4200 mg/d, days 1-14 and oxaliplatin 250 mg/d, 3-wk intervals) in combination with bevacizumab (anti-vascular endothelial growth factor antibody, 700 mg/d, 3-wk intervals) and IRIS (S-1, 120 mg/d, days 1-14 and irinotecan, 240 mg/d, 2-wk intervals) as the first- and second-line treatments, respectively. During these treatments, the skin lesion of psoriasis showed neither significant worsening nor improvement.

At 29 mo after the initial diagnosis, the patient commenced third-line treatment with cetuximab antibody (a human-and-mouse chimeric anti-EGFR monoclonal antibody, 800 mg on day 1, 500 mg on day 8, and once a week thereafter). Notably, seven days after the first administration of cetuximab, the psoriasis showed significant improvement (Figure 1). Subsequently, an allergic reaction necessitated replacement of the cetuximab with panitumumab (a fully human anti-EGFR monoclonal antibody, 600 mg/d, 2-wk intervals); however, the skin lesion improvement was maintained. The third-line treatment was continued for six months and the best response

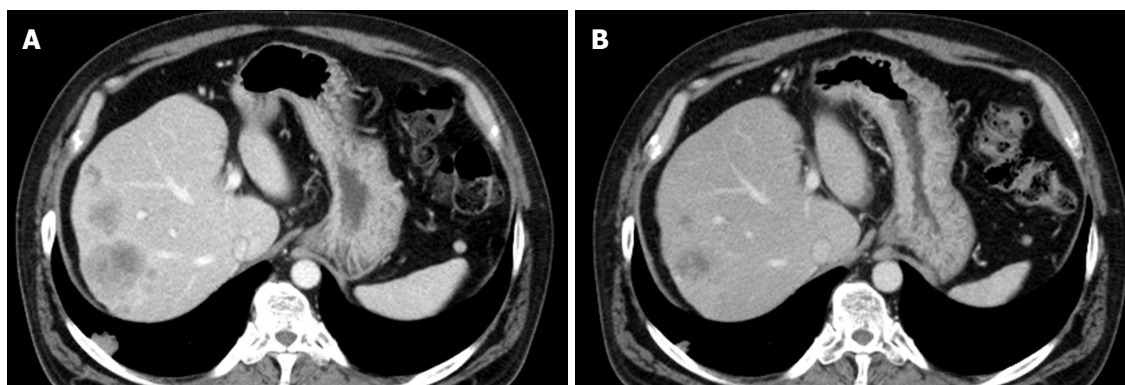


Figure 2 Effect of anti-epidermal growth factor receptor antibody on liver metastatic lesions of colon cancer. A: Enhanced abdominal computed tomography showed metastatic lesions throughout the liver, including in the right posterior segment; B: The best response was partial response, with eventual regrowth.

was partial response (Figure 2). The patient showed grade1 folliculitis on his face as an adverse effect of cetuximab or panitumumab, although it did not require treatment. The psoriasis symptoms worsened after discontinuation of the anti-EGFR monotherapy, and the patients died five months later.

DISCUSSION

EGFR is expressed in epithelial cells including keratinocytes and hair follicles, and plays a critical role in the development and function of normal skin^[9,10]. In addition, skin lesions of psoriasis patient have revealed EGFR overexpression^[3,4,11], suggesting sustained keratinocyte viability due to EGFR-mediated hyperstimulation as an underlying mechanism in the development of psoriatic skin lesions. In support of this hypothesis, EGFR tyrosine kinase inhibitor used for the treatment of lung cancer induced some improvement in psoriasis skin lesions^[12], while Trivin *et al*^[5] reported the regression of psoriasis after combination therapy with cetuximab and 5-fluorouracil/folinic acid for metastatic colon cancer. Although this report could not eliminate the possible effect of 5-fluorouracil, Neyns *et al*^[6] later reported marked improvement of psoriasis during cetuximab monotherapy for the treatment of metastatic colon cancer.

Contrarily and surprisingly, Mas-Vidal *et al*^[8] reported a case of psoriasis developing 12 d after the initiation of cetuximab treatment and resolving with discontinuation of treatment, and accordingly, they suggested a causal association between cetuximab and psoriasis. Similarly, Zorzou *et al*^[13] reported the unexpected exacerbation of psoriasis after EGFR tyrosine kinase inhibitor treatment in patients with squamous cell lung cancer. Although a mechanism for the development and exacerbation of psoriasis with these treatments remains unclear^[8], thresholding of EGFR signal blockade could induce alterations in the skin immune system and/or to the activation of alternative signaling pathways for psoriatic keratinocyte proliferation in selected patients. In any case, these inconsistent reports necessitate further

studies and patient data collection focusing on the link between psoriasis vulgaris and EGFR.

Anti-EGFR antibody is effective in treating KRAS wild-type advanced colorectal cancer and various kinds of solid tumors. However, dermatological toxicities such as papulopustular rash occur in 70%-90% of patients, with the consequent physical and psychosocial discomfort potentially resulting in discontinuation of treatment^[14]. Therefore, anti-EGFR antibodies are not generally used in patients with concomitant chronic skin disease, despite the lack of definitive evidence suggesting adverse skin effects. The case reported herein now presents important data and supports the previous experience that the use of cetuximab and/or panitumumab for colorectal cancer is not necessarily contraindicated in patients with psoriasis vulgaris^[6], and indeed, suggests that such therapy could be beneficial for the treatment of psoriasis vulgaris. Importantly, the currently prevalent treatment with anti-EGFR antibodies is unlikely to “cure” psoriasis. Therefore, the case presented by Trivin *et al*^[5] is quite attractive in that complete remission of psoriasis was sustained for more than 6 months after discontinuation of the treatment.

In conclusion, we experienced a patient with psoriasis vulgaris whose symptoms lessened during treatment with anti-EGFR antibody monotherapy for metastatic colon cancer. We hope that this case report serves to further clarify the pathogenesis of psoriasis vulgaris. In addition, patients with concomitant skin disease should still be considered for anti-EGFR antibody therapy.

COMMENTS

Case characteristics

A 55-year-old male patient with long-standing psoriasis vulgaris was referred to our department for the treatment of colon cancer.

Clinical diagnosis

Metastatic colon cancer and psoriasis vulgaris.

Imaging diagnosis

Computed tomography revealed metastatic colon cancer, and dermatological inspection showed typical skin lesions of psoriasis vulgaris.

Pathological diagnosis

Postoperative pathological examination revealed typical sigmoid colon cancer (not described in the manuscript).

Treatment

Anti-epidermal growth factor receptor (EGFR) antibody was effective against both metastatic colon cancer and psoriasis vulgaris.

Related reports

The effect of anti-EGFR antibody on psoriasis vulgaris is not well understood.

Experiences and lessons

For the treatment of metastatic colon cancer, anti-EGFR antibody therapy should be still considered in patients with psoriasis vulgaris and/or chronic dermatological diseases.

Peer-review

This report describes a quite rare case of colon cancer and concomitant psoriasis vulgaris successfully treated with anti-EGFR antibody.

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Cytomegalovirus colitis followed by ischemic colitis in a non-immunocompromised adult: A case report

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Ethics approval: This case was reviewed and approved by the Aomatsu Memorial Hospital Institutional Review Board.

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Abstract

We report a rare case of cytomegalovirus (CMV) colitis followed by severe ischemic colitis in a non-immunocompromised patient. An 86-year-old woman was admitted after experiencing episodes of vomiting and diarrhea. The next day, hematochezia was detected without abdominal pain. The initial diagnosis of ischemic colitis was based on colonoscopy and histological findings. The follow-up colonoscopy revealed a prolonged colitis. Immunohistochemical staining detected CMV-positive cells following conservative therapy. Intravenous ganciclovir therapy led to successful healing of ulcers and disappearance of CMV-positive cells. The prevalence of CMV infection is common in adults. CMV colitis is relatively common in immunocompromised patients; however, it is rare in immunocompetent patients. In our case, CMV infection was allowed to be established due to the disruption of the colonic mucosa by the prior severe ischemic colitis. Our experience suggests that biopsies may be necessary to detect CMV and the prompt management of CMV colitis should be instituted when intractable ischemic colitis is observed.

Key words: Cytomegalovirus infection; Colitis; Ischemic colitis; Non-immunosuppression; Infectious disease

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Core tip: Cytomegalovirus colitis is common in immunocompromised patients but rare in immunocompetent patients. In cases where ischemic colitis is prolonged, it is important to consider cytomegalovirus colitis. This case report not only represents the colonoscopy and

pathological findings in immunocompetent patients, but also applies the method of diagnosing and treating immunocompetent patients.

Hasegawa T, Aomatsu K, Nakamura M, Aomatsu N, Aomatsu K. Cytomegalovirus colitis followed by ischemic colitis in a non-immunocompromised adult: A case report. *World J Gastroenterol* 2015; 21(12): 3750-3754 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3750.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3750>

INTRODUCTION

Cytomegalovirus (CMV) is a well-recognized pathogen, with 40%-100% of the general population showing prior exposure as per serology^[1]. However, most infected adults show no symptoms or occasionally show self-limiting infectious mononucleosis^[2]. Gastrointestinal CMV disease usually occurs in immunocompromised patients^[3]. CMV colitis is rarely reported in non-immunocompromised patients^[4]. Herein, we report a rare case of CMV colitis in an immunocompetent patient followed by ischemic colitis and successfully managed by antiviral therapy.

CASE REPORT

An 86-year-old woman was admitted following one day episodes of vomiting (three times) and diarrhea (three times). The patient denied any recent travel or changes in medication usage, including antibiotics and non-steroidal anti-inflammatory drugs. The patient had a history of diabetes mellitus and hypertension but no history of inflammatory bowel disease (IBD), renal failure, valvular disease or coronary artery disease. General examination revealed a malnourished woman with a body mass index of 19.1 kg/m². Her vitals included a body temperature of 36.5 °C, a heart rate of 81 bpm and blood pressure of 82/49 mmHg. Other findings were unremarkable, including a soft abdomen and normal active bowel sounds.

Laboratory results for white blood cells, hemoglobin, hematocrit, platelets, serum creatinine, total protein, albumin, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were within their normal range. C-reactive protein, lactate dehydrogenase, creatinine phosphokinase, serum creatinine (Cre) and fasting blood sugar were slightly elevated at 0.45 mg/dL, 346 U/L, 241 U/L, 0.90 mg/dL and 190 mg/dL, respectively. The prothrombin time was within the normal range, while the activated partial thromboplastin time was short at 20.5 s. Stool culture at the first visit was not performed.

After admission, the patient had a broad-spectrum antibiotic *via* instillation administered. The next day, diarrhea and vomiting subsided. However, hematochezia was detected without abdominal pain

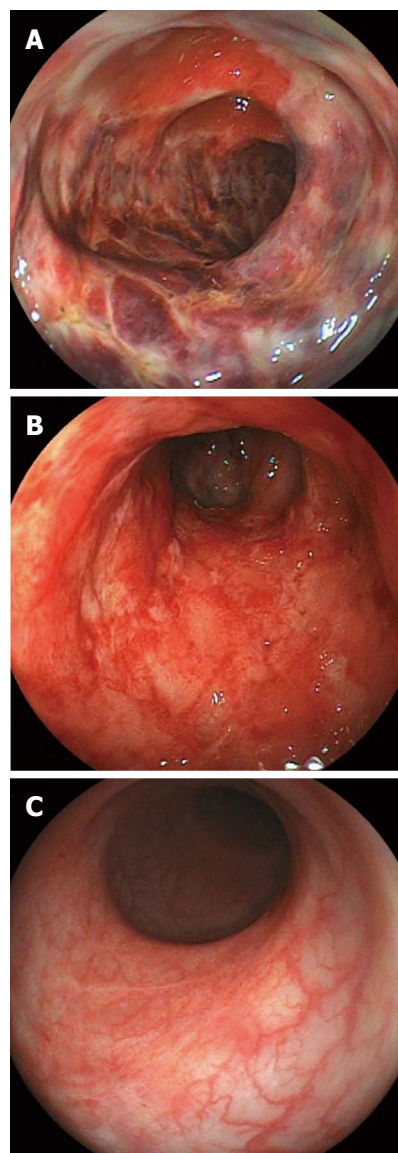


Figure 1 Endoscopic findings. A: First colonoscopy (day 4), mucosal hyperemic change with edema, erosion and ulcerations and hemorrhagic friable mucosa from the descending to sigmoid colon; B: Follow-up colonoscopy after conservative therapy (day 25); ulcerations remain; C: Follow-up colonoscopy after ganciclovir therapy (day 46); ulcerations have healed.

the next day. On day 4, colonoscopy revealed acute, inflamed and friable mucosal changes as well as ulcers spread widely and annularly from the transverse colon to sigmoid colon (Figure 1A). Biopsy specimens taken from the ulcers showed acute exudative colitis compatible with ischemic colitis. After administration of the antibiotic fosfomycin calcium hydrate (2 g/d), a high fever and an elevated white blood cell count were detected on day 11. Consequently, the treatment was changed to 2 g/d of cefotiam hydrochloride. A test for *Clostridium difficile* toxin in the stool was negative.

Follow-up colonoscopy on day 25 demonstrated stenosis at the splenic flexure, with a shallow and circumferential ulcer from the descending colon to sigmoid colon remaining (Figure 1B). The stenosis segment, estimated with a contrast medium, appeared

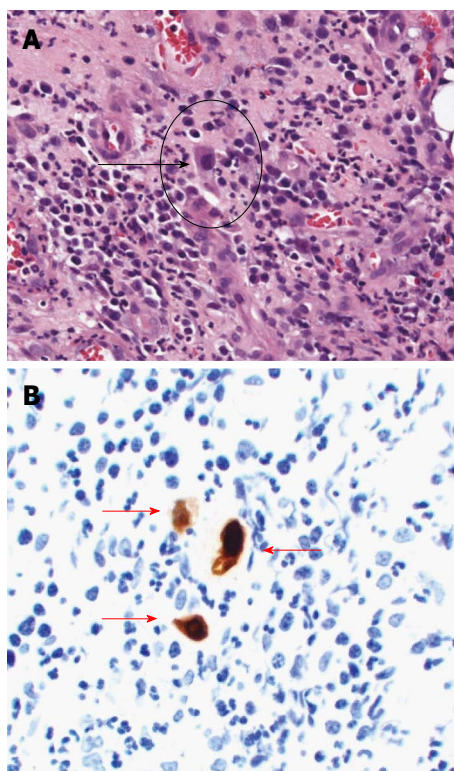


Figure 2 Pathological examinations following hematoxylin-eosin and immunohistochemical staining. A: White arrow shows cytomegalovirus inclusion bodies (HE staining, $\times 200$); B: Red arrows show cytomegalovirus-positive cells (immunohistochemical staining, $\times 200$).

short and incomplete. Serum CMV IgG and IgM were seropositive and seronegative, respectively. CMV pp65 antigen was negative. A quantitative polymerase chain reaction (PCR) assay to detect CMV was not performed. Histopathological evaluation of the biopsy specimens from the colonic mucosa revealed granulation tissue with dilatation of blood capillaries. Enlarged cells with viral inclusions were observed in the biopsy specimens (Figure 2A). Immunohistochemical findings with an anti-CMV antibody showed positively stained cells (Figure 2B). After diagnosis of CMV colitis, the patient was treated with intravenous ganciclovir at 500 mg/d for seven days. A follow-up colonoscopy on day 46 showed disappearance of the colonic ulcers (Figure 1C) and stenosis persisting in the descending colon. A biopsy demonstrated disappearance of the CMV inclusion bodies and the CMV-positive cells. Figure 3 shows the clinical course of the case. The follow-up colonoscopy at five months showed normal mucosa, stenosis in the descending colon and the presence of inflammatory polyps. The patient is carefully monitored once a month, has no trouble evacuating the bowels and remains asymptomatic in November 2014.

DISCUSSION

CMV is a double-stranded DNA virus belonging to the herpes virus family. CMV infections are common worldwide due to excretion of CMV in bodily fluids,

including saliva, respiratory secretions, urine, blood, breast milk and semen, and is transmitted by close personal contact. The prevalence of CMV infections in the general adult population is approximately 40%-100%^[1]. In normal hosts, primary infection is usually asymptomatic but can sometimes result in a syndrome similar to infectious mononucleosis, accompanied by symptoms such as fever, myalgia, cervical lymphadenopathy and elevated liver enzymes^[4]. Several reports have shown that CMV can cause severe disease in immunocompromised patients; for instance, patients that have been treated with steroids, have renal failure, acquired immune deficiency syndrome, cancer or IBD, or have undergone bone marrow or solid tumor transplants^[3,5-7]. A meta-analysis of CMV colitis among immunocompetent patients was performed by Galiatsatos *et al.*^[4] and included 44 cases over 23 years. Only three case reports have been written by Japanese researchers on CMV colitis after ischemic colitis in immunocompetent patients.

In our case, abdominal pain, a typical symptom of ischemic colitis, was not detected. Although the symptoms with no abdominal pain are atypical in ischemic colitis, colonoscopy results, location of the disease and biopsy results were in accordance with ischemic colitis. Patients with diabetes mellitus often have a nerve disorder and at times do not feel pain, even in the presence of myocardial infarction. Our patient was old and also had a history of diabetes mellitus, providing a potential explanation for why abdominal pain was not detected.

CMV target organs include the gastrointestinal tract, lung, retina, liver and central nervous system^[4]. CMV can infect the gastrointestinal tract from the esophagus to the rectum, with the most frequent site in immunocompetent patients being the colon. These data are in concordance with those reported by Galiatsatos *et al.*^[4]. The symptoms of CMV colitis include abdominal pain, anorexia, malaise, nausea, vomiting, diarrhea and bleeding^[5]. Colonic perforation, although uncommon (about 1% of cases), is a potentially fatal complication in these patients.

Colonoscopy can reveal a range of CMV colitis related findings, such as shallow, erythematous erosions or localized ulcers and, less commonly, plaques, nodules and polyps. Biopsies are essential because similar findings can be present in other types of colitis. Pathological examination of the involved gastrointestinal site typically reveals diffuse ulcerations and necrosis with scattered CMV inclusions that play an active role in damaging the colonic mucosa, primarily as a result of CMV vasculitis^[6]. These characteristics are consistent with our patient's pathological findings.

Previously, diagnosing CMV infection required at least one of the following laboratory methods: serology, direct detection of CMV pp65 antigen in the blood or CMV culture. Increase in the serum anti-CMV IgM and IgG levels indicates a recent or a past

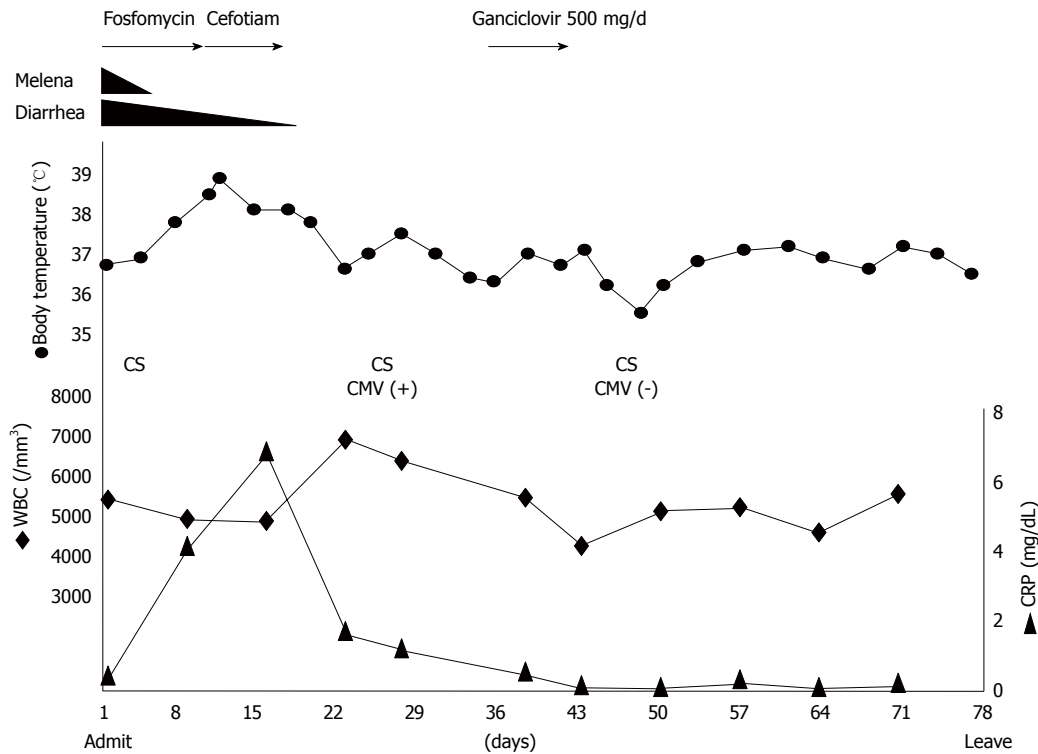


Figure 3 Clinical course of this case. CS: Colonoscopy; CMV: Cytomegalovirus; CRP: C-reactive protein; WBC: White blood cells.

CMV infection, respectively. Although anti-CMV IgG and IgM serum levels have been previously widely used at diagnosis of CMV infection, serology and virus detection tests are not very sensitive and may not detect the virus despite disease progression. Culturing CMV from body fluids or tissue samples is another method, although a time-consuming one with low sensitivity. Hematoxylin-eosin (HE) staining and immunohistochemical staining appear to be more sensitive than serological or virus isolation tests^[2]. The HE sensitivity is 10%-87%^[8] and CMV inclusion bodies can be found in biopsy specimens. Recent usage of PCR assay to detect CMV-DNA has led to successful diagnosis; however, the technology is not commonly available^[9]. Therefore, the final diagnosis relies on histological findings, including the presence of CMV inclusion bodies, and the immunohistochemistry or hybridization results. In our case, there was an increase in the levels of anti-CMV IgG antibodies despite CMV pp65 antigen being negative. Histological findings showed CMV inclusion bodies detected in HE staining. Immunohistochemical staining was positive. PCR detection was not performed.

Two theories, the primary and secondary, have been proposed regarding the mechanism of the pathogenesis of CMV colitis. The primary theory is that the CMV can proliferate in the vascular endothelial cells, leading to vasculitis and small vessel thrombosis with local ulceration^[7]. Furthermore, large vessel vasculitis may lead to thrombosis and subsequently to ischemic colitis. According to the secondary theory, previous diseases, such as ischemic colitis

or IBD, destroy the colonic mucosa, leading to local immunosuppression. CMV infection may occur under such mucosal conditions. In the case of our patient, severe ischemic colitis preceded CMV infection that was allowed to be established due to the disruption of the colonic mucosa by the prior ischemic colitis. CMV inclusion bodies were detected at the second follow-up colonoscopy but not at the first colonoscopy.

Ganciclovir is currently a standard anti-viral drug for the treatment of CMV infection. Both ganciclovir and foscarnet have shown to significantly improve the prognosis of CMV disease of the gastrointestinal tract^[6]. Unfortunately, ganciclovir can lead to serious side effects, including myelosuppression, central nervous system disorders, hepatotoxicity and nephrotoxicity^[2]. Despite these serious side effects, Eddleston recommended antiviral therapy for immunocompetent patients due to poor prognosis in the absence of antiviral treatment^[10].

In our patient, CMV colitis prolonged ischemic colitis and colonoscopy showed severe colitis symptoms. We correctly diagnosed CMV colitis with positive immunohistochemical staining. Implementation of intravenous ganciclovir therapy led to considerable healing and disappearance of positive immunohistochemical staining. No ganciclovir-related side effects were detected. Concerning the stenosis detected by colonoscopy, we plan to operate or place a stent in this stenosis when complete obstruction is noticed at this area.

A secondary CMV infection must be suspected when prolonged ischemic colitis is noted following

conservative therapy. Biopsies or PCR tests may be necessary to diagnose CMV colitis. CMV colitis may be the cause of more cases of intractable ischemic colitis than are currently being documented.

COMMENTS

Case characteristics

An 86-year old woman presented with one day episodes of vomiting and diarrhea.

Clinical diagnosis

A day after admission, diarrhea and vomiting decreased but diarrhea changed into hematochezia without abdominal pain.

Differential diagnosis

Colon cancer, ischemic colitis, bleeding of diverticulum.

Laboratory diagnosis

C-reactive protein, 0.45 mg/dL; lactate dehydrogenase, 346 U/L; creatinine phosphokinase, 241 U/L; creatinine, 0.90 mg/dL; fasting blood sugar, 190 mg/dL; complete blood counts and liver functions were within normal range.

Imaging diagnosis

The first colonoscopy showed acute, inflamed, friable mucosal changes, while the second colonoscopy detected that these findings were prolonged.

Pathological diagnosis

Colonoscopy and biopsy revealed cytomegalovirus (CMV) colitis followed by ischemic colitis, CMV positive.

Treatment

The patient was treated with intravenous ganciclovir.

Related reports

CMV infection is common in adults and most infected adults do not show symptoms. However, gastrointestinal CMV disease usually occurs in immunocompromised patients.

Term explanation

"Immunocompetent" means patients have normal immunity.

Experiences and lessons

This case report suggests that a biopsy may be necessary to detect CMV when intractable ischemic colitis is observed.

Peer-review

This case report demonstrates application of the clinical and endoscopic methods to diagnose cytomegalovirus colitis and strongly recommends prompt

management of cytomegalovirus in immunocompetent patients.

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Liver transplantation for recurrent posthepatectomy malignant hepatic angiomyolipoma: A case report

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cells, adipose tissue, and vessels, and are commonly found in the kidney and occasionally in the liver. The preoperative diagnosis of hepatic AML is primarily made from imaging and fine-needle aspiration biopsy results, though limited experience for such diagnoses can result in misdiagnosis. Some uncommon features of hepatic AML have been reported in the literature without an objective or qualitative consensus. As the majority of cases are benign, conservative treatment of AMLs is recommended. However, in rare cases, liver transplantation has been implemented. Only five cases of malignant hepatic AML have been reported. We report a rare case of recurrent posthepatectomy malignant hepatic AML that was misdiagnosed as liver cancer in a 37-year-old woman, which was treated by liver transplantation. The imaging and pathologic findings are presented in order to provide a more concise description to aid in future diagnoses.

Key words: Angiomyolipoma; Hepatectomy; Malignant; Liver transplantation; Recurrence

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Core tip: Hepatic angiomyolipomas (AMLs) are typically benign tumors, with rare reports of malignant cases. Because there is a lack of experience for preoperative diagnosis, hepatic AMLs can easily be misdiagnosed. This case report not only presents some characteristics of hepatic AML and diagnostic indicators of malignancy, but also describes the successful treatment with liver transplantation.

Wang WT, Li ZQ, Zhang GH, Guo Y, Teng MJ. Liver transplantation for recurrent posthepatectomy malignant hepatic angiomyolipoma: A case report. *World J Gastroenterol* 2015; 21(12): 3755-3758 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3755.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3755>

Abstract

Hepatic angiomyolipomas (AMLs) are typically benign tumors containing varying amounts of smooth muscle

INTRODUCTION

Angiomyolipoma (AML) is a rare mesenchyme-derived neoplasm that most commonly occurs in the kidney, but can also be found in the liver. Hepatic AML was first reported by Ishak^[1] in 1976, and has since been considered a benign tumor requiring conservative treatment. However, a malignant case with evidence of recurrence has been more recently reported^[2]. The first case of liver transplantation for hepatic AML was reported in 2010^[3], and only two additional cases have since been described^[3,4]. We report a case of a Chinese woman with recurrent posthepatectomy malignant hepatic AMLs who required liver transplantation. The details of this case will help for future diagnosis and treatment of malignant hepatic AML.

CASE REPORT

A 37-year-old Chinese woman had been diagnosed with liver cancer two years before. An abdominal enhanced computed tomography (CT) scan in 2011 showed a hepatic mass with early-phase hyperattenuation and late-phase hypoattenuation, measuring 7 cm × 9 cm in the left lobe (Figure 1A-C). Magnetic resonance imaging revealed a mass with hypointensity on T1-weighted, hyperintensity on T2-weighted, and hyperintensity on diffusion-weighted images (Figure 1D and E), and the patient underwent hepatic resection. Pathologic evaluation of the specimen indicated that the tumor was a hepatic AML.

The patient was subsequently lost to follow-up after surgery, during which time she gave birth to a baby and was not diagnosed during health checkups. She was admitted to our hospital in 2014 for further examination of a hepatic tumor that had been found incidentally during examinations. There was no medical history of other malignant diseases, and the blood exams were negative for tumor markers and viral markers. Enhanced CT showed two hepatic masses 13.0 cm × 12.0 cm and 2.3 cm × 1.8 cm in the right lobe (Figure 1F and G). On angiography, the tumor was shown as a circumscribed hypervascular mass (Figure 1H). After transcatheter arterial chemoembolization, liver transplantation was performed.

The two tumors appeared gray and white; the larger tumor measured 15 cm in diameter (Figure 2A), while the smaller one measured 3 cm in diameter. The diagnosis of hepatic AML was confirmed by immunohistochemical examination. Most of the epithelioid cells were immunopositive for human melanoma black-45 (Figure 2B), and there was no evidence of vascular invasion or extrahepatic metastasis. Nuclear atypia and Ki-67 antigenicity (4%) were found (Figure 2C). Additional indicators of

malignancy were found (Figure 2D-F), and the final pathologic diagnosis was malignant hepatic AML. The patient recovered well and is currently healthy.

DISCUSSION

Of the 200 cases of hepatic AML reported, only five have described malignancies^[2-5], and three with hepatic recurrence^[5]; this is the sixth reported case of malignant hepatic AML. Most of the patients were asymptomatic, and preoperatively diagnosed from imaging and fine-needle aspiration biopsy findings. Because of the rarity of objectively malignant cases, it is difficult to distinguish benign from malignant hepatic AML, and subsequently, to determine whether to treat the patient conservatively or with surgery. Conservative treatment has been recommended for the patients with: (1) tumors smaller than 5 cm; (2) hepatic AML determined by fine-needle aspiration biopsy; (3) good compliance; and (4) hepatitis virus^[6]. In contrast, surgery is the only accepted treatment for patients with: (1) multiple or large, unresectable lesions; (2) serious life-threatening complications; and (3) tumors with a malignant potential, for whom liver transplantation is available^[2,7-9].

Liver transplantation has been proposed for the treatment of benign liver diseases^[10], but only approximately 50 cases have been reported worldwide. However, there are only two reported cases of liver transplantation for AML. The first case was reported by Dumortier *et al.*^[3], describing a patient who died of multi-organ lesions after liver transplantation. The other report by Vagefi *et al.*^[4] involved a 20 cm mass, for which the authors suggested liver transplantation referral in similar cases because the lesions are not amenable to resection. However, the tumors in these two cases were consistent with benign hepatic AMLs.

A model was proposed for predicting malignant behavior of renal AMLs, incorporating the following features: ≥ 2 mitotic figures per 10 high-powered fields, atypical epithelioid cells, atypical mitotic figures and necrosis; the preoperative diagnostic accuracy rate was 78%^[6]. In accordance, the case presented here also showed atypical epithelioid cells, atypical mitotic figures and necrosis. We also observed cells positive for human melanoma black-45 and Ki-67, which have been suggested as indicators of hepatic AML malignancy by Mizuguchi *et al.*^[7]. However, whether the predictive model of renal AML is applicable for hepatic AML requires further research.

Although malignant hepatic AML is extremely rare, a possible diagnosis should not be ignored. As liver transplantation has been the method of treatment for hepatic AML, preoperative evaluation and discussion must be made with caution.

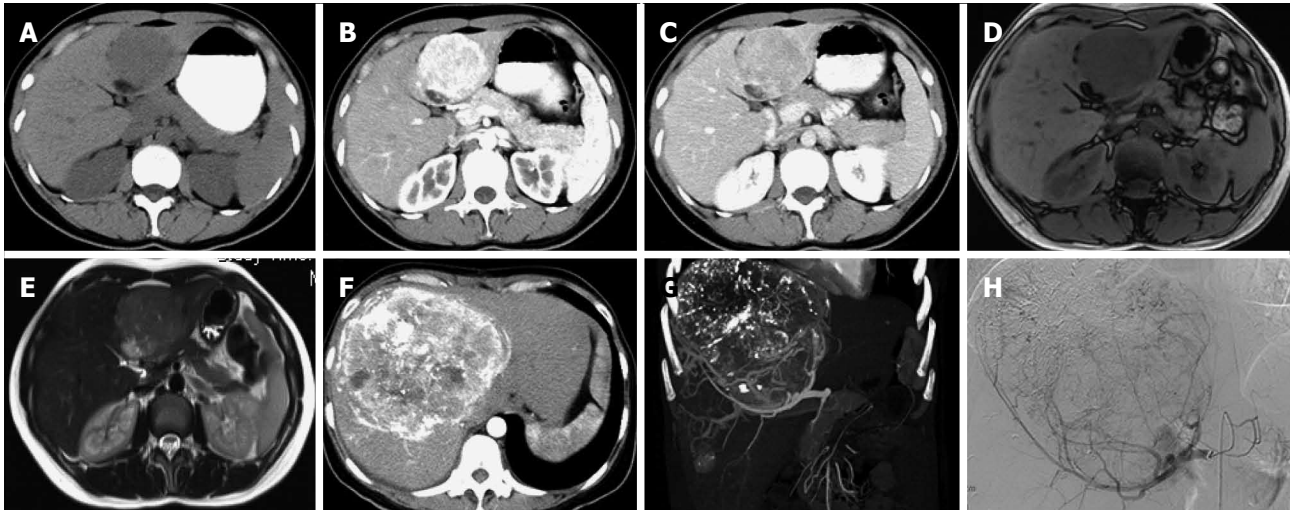


Figure 1 Imaging findings. A: The tumor was hypoechoic on computed tomography (CT); B, C: Enhanced CT showed a tumor (7.0 cm × 9.0 cm) with early-phase hyperattenuation and late-phase hypoattenuation; D, E: Magnetic resonance imaging showed a tumor with hypointensity on T1-weighted, hyperintensity on T2-weighted; F, G: Enhanced CT showed two hepatic masses (13.0 cm × 12.0 cm and 2.3 cm × 1.8 cm) in the right lobe; H: Angiography revealed a circumscribed hypervascular mass.

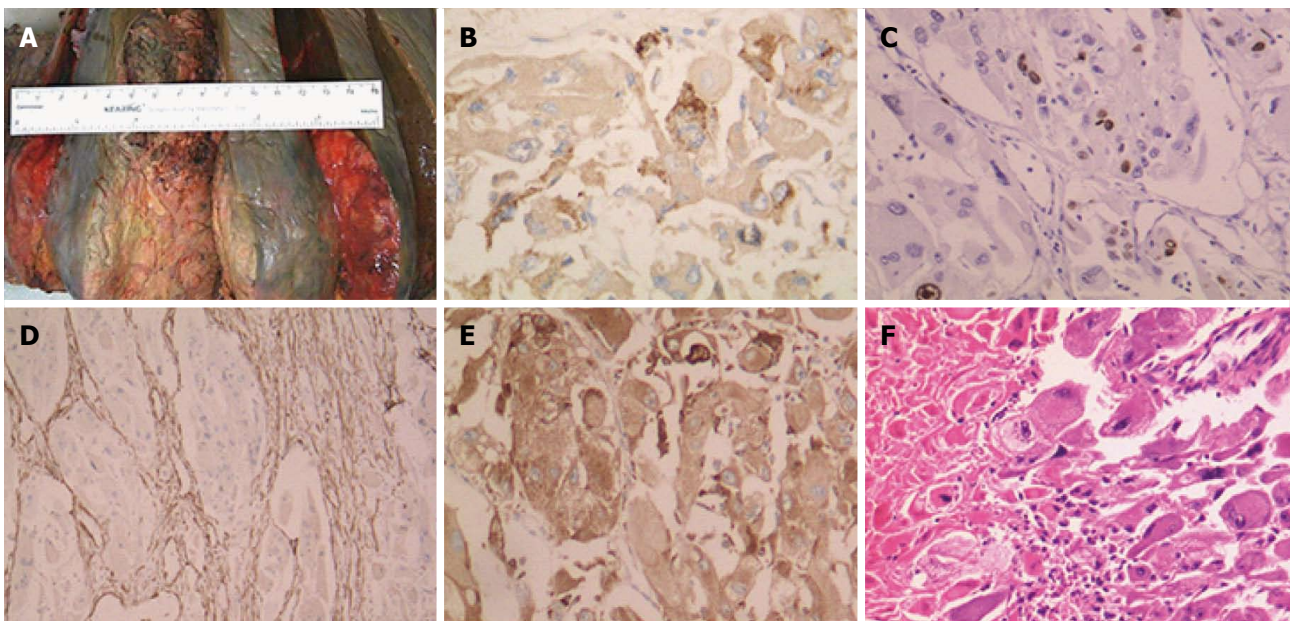


Figure 2 Pathologic findings. A: The tumor occupied a large area of the liver; The tumor was immunopositive for B: Human melanoma black-45 (20 ×); C: Ki-67 (10 ×); D: Smooth muscle actin (10 ×); E: CD68 (20 ×); F: Hematoxylin and eosin staining showed neoplasm necrosis (4 ×).

COMMENTS

Case characteristics

A 37-year-old woman with recurrent posthepatectomy malignant hepatic angiomyolipoma (AML) without symptoms was misdiagnosed with liver cancer.

Clinical diagnosis

Recurrent posthepatectomy malignant hepatic AML.

Differential diagnosis

Hepatic malignant tumor; Focal nodular hepatic fatty infiltration; Hepatic hemangioma.

Laboratory diagnosis

WBC, 8.20 k/μL; hemoglobin, 12.10 g/dL; alpha-fetoprotein 1.31 ng/mL; liver function tests were within normal limits.

Imaging diagnosis

Enhanced computed tomography showed two hepatic masses with early-phase hyperattenuation and late-phase hypoattenuation, measuring 13.0 cm × 12.0 cm and 2.3 cm × 1.8 cm in the right lobe; upon angiography, the tumor was shown as a circumscribed hypervascular mass.

Pathological diagnosis

Benign AMLs have little or no mitotic activity, which was present in this case (human melanoma black-45-, smooth muscle actin- and CD68-positive).

Treatment

The patient received a liver transplantation.

Related reports

Malignant hepatic AML is extremely rare, and there are no proposed models for predicting malignancy.

Term explanation

AML is a rare mesenchyme-derived neoplasm, typically seen as benign tumors in the kidney, but can also be found in the liver.

Experiences and lessons

This case report not only presents the features of malignant hepatic AML, but also applies liver transplantation as a treatment method.

Peer-review

The authors of the case report entitled "Liver transplantation for recurrent posthepatectomy malignant hepatic AML" present a problem of the use of liver transplantation as a method of treatment of liver malignant tumors. This case report is well written.

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Gastritis cystica profunda in a previously unoperated stomach: A case report

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Author contributions: Teng LS designed the report; Chen ST collected the patient's clinical data; Guo LW and Yu XF analyzed the data and wrote the paper.

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Abstract

Gastritis cystica profunda is a relatively rare disease, usually observed at anastomotic sites in stomachs of patients that have undergone gastric procedures. We present the rare case of an elevated lesion in the anterior wall of the gastric antrum of a 43-year-old Chinese woman who had never undergone gastric surgery and had no gastrointestinal tract symptoms. Although the physical examination and laboratory data showed no abnormalities, endoscopic ultrasonography revealed an anechoic cystic structure. Abdominal

computed tomography and magnetic resonance imaging showed the gastric wall of the greater curvature of the antrum was markedly and irregularly thickened, and mild to moderate enhancement was observed around the lesion with no enhancement in the central portion, suggestive of a gastrointestinal stromal tumor. The patient underwent a distal gastric resection of the 2.5 cm × 1.5 cm lesion. A postoperative pathologic examination showed dilated cystic glands in the muscularis mucosa and submucosal layers and erosion of the mucosal surface of the tumor, confirming the diagnosis of gastritis cystica profunda without malignancy.

Key words: Gastritis cystica profunda; Hyperplastic polyp; Stomach

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Core tip: Gastritis cystica profunda is a rare disease characterized by polypoid hyperplasia and cystic dilatation of the gastric glands extending into the submucosa of the stomach. It is typically only found in the stomach after gastric surgery, however, we encountered a rare case of gastritis cystica profunda in a 43-year-old Chinese woman who had never undergone gastric surgery. The elevated lesion in the anterior wall of the gastric antrum was discovered by endoscopic ultrasonography, and marked and irregular thickening of the gastric wall was observed with computed tomography and magnetic resonance imaging.

Yu XF, Guo LW, Chen ST, Teng LS. Gastritis cystica profunda in a previously unoperated stomach: A case report. *World J Gastroenterol* 2015; 21(12): 3759-3762 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i12/3759.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i12.3759>

INTRODUCTION

Gastritis cystica profunda (GCP) is a relatively rare, benign disease characterized by polypoid hyperplasia and cystic dilatation of the gastric glands that extend into the submucosa of the stomach^[1]. GCP can present as upper gastrointestinal symptoms, such as upper abdominal pain, acid reflux, nausea, anorexia, or bleeding, though some patients may experience no symptoms at all^[2], and can cause massive upper gastrointestinal hemorrhage and gastric outlet obstruction in some cases^[3-5]. GCP usually emerges as giant gastric folds, submucosal tumors, or isolated polyps and typically occurs at anastomotic sites of previous surgeries^[6]. Here we report a unique case of GCP in an asymptomatic patient who had no history of gastric surgery.

CASE REPORT

A 43-year-old Chinese woman was admitted to our hospital for an abnormality of the stomach that was discovered during a routine medical check-up. Gastroscopy revealed a mass-like lesion protruding into the gastric lumen (Figure 1). She had no gastrointestinal symptoms and no history of abdominal surgery. Physical examination and laboratory data showed no abnormalities. Endoscopic ultrasonography revealed an anechoic cystic structure in the irregularly thickened stomach wall of the greater curvature of the antrum (Figure 2). Additional abdominal computed tomography and magnetic resonance imaging also showed that the gastric wall was markedly and irregularly thickened, and enhanced scanning showed mild to moderate enhancement around the lesion, suggestive of a gastrointestinal stromal tumor (Figure 3).

A distal gastric resection (Billroth I) was performed to remove the 2.5 cm × 1.5 cm tumor, which had an eroded mucosal surface. A histologic examination revealed dilated cystic glands in the muscularis mucosa and submucosa (Figure 4), consistent with a diagnosis of GCP without malignancy.

DISCUSSION

The etiology and pathogenesis of GCP have not been established, however, lesions are often accompanied by severe diffuse chronic gastritis^[7] and *Helicobacter pylori* infection^[8]. It is thought that inflammation causes the erosion of the muscularis mucosa, consequently leading to the formation of submucosal cysts. The potassium voltage-gated channel subfamily E member 2 (*KCNE2*), an essential subunit of apical potassium channels in parietal cells, may also be implicated, as knockout mice exhibit a severe gastric preneoplastic phenotype^[9], and the expression of *KCNE2* is negatively correlated with gastric cancer formation in humans^[10]. GCP is also considered to be

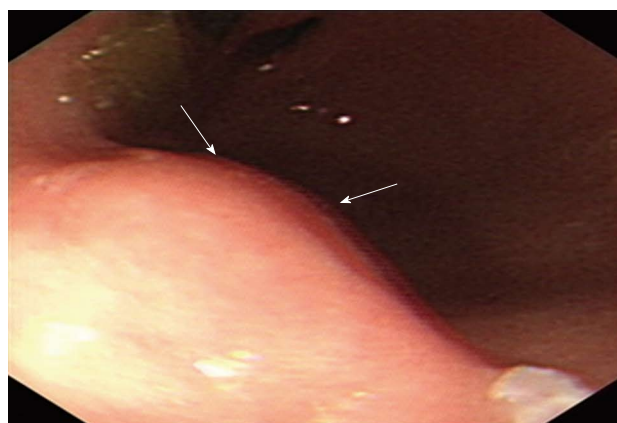


Figure 1 Gastroscopy. An elevated nodular lesion (arrows) with smooth surfaces was observed in the anterior portion of the gastric antrum.

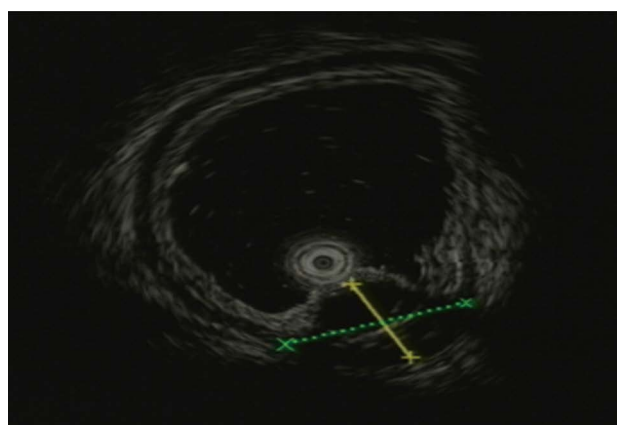


Figure 2 Endoscopic ultrasonography. An anechoic mass with an irregularly thickened wall was observed in the submucosal lining of the stomach.

precancerous^[8], as a few cases have been associated with gastric cancers^[11-13] and gastric carcinoma with lymphoid stroma^[14]. There is also evidence that the development of GCP is influenced by infection with the Epstein-Barr virus, which was observed in both dysplastic and carcinoma areas^[14], and is more prevalent in gastric cancer tissues from patients with GCP^[11].

As GCP is typically found at sites of previous surgeries, it was suggested that mucosal injury caused by surgery or the suture technique itself promotes mucosal prolapse and herniation of glands into the submucosa^[15,16]. Interestingly, the patient in the present case had no history of abdominal surgery, thus additional factors must have contributed. Mukaisho *et al*^[17] found GCP-like mucosal changes in an animal model of duodenal reflux, of which 76.2% developed adenocarcinomas at week 80. Repeated erosion of the stomach lining may give rise to heterotopic cysts, the surface mucosa of which may be more prone to further erosion. Additionally, the regeneration of mucosal epithelium may lead to the development of carcinoma^[8]. Indeed, GCP lesions may be pre-cancerous, as Ochiai *et al*^[13] found that cell kinetics

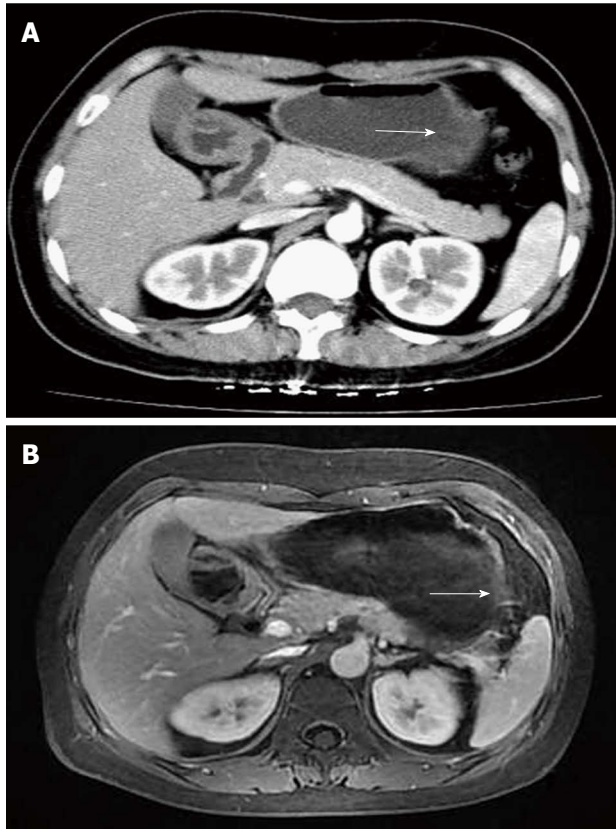


Figure 3 Radiology. A: Computed tomography; B: Magnetic resonance imaging of the patient in a prone position showed that the gastric wall of the stomach at the greater curvature of the antrum was markedly and irregularly thickened, with mild to moderate enhancement (arrow).

were accelerated and gene mutations were enhanced in GCP lesions, and foveolar glands in GCP can be hyperplastic and highly proliferative^[18]. Therefore, patients with non-malignant GCP should continue to be monitored.

The main treatment for GCP is surgical removal of the lesion, while minimizing bile reflux into the stomach. Due to the premalignant nature of the disease, histopathologic evaluation of the lesion is recommended. In addition, GCP recurrence after surgical resection has been reported^[19]. Therefore, careful long-term follow-up is needed. The case reported here demonstrates that GCP is not limited to patients who have previously undergone gastric surgery, and should therefore be considered for cases with abnormalities of the stomach mucosa.

COMMENTS

Case characteristics

A 43-year-old Chinese woman who had no history of gastric surgery presented with an abnormality of the stomach by gastroscopy during a routine medical check-up.

Clinical diagnosis

Endoscopic ultrasonography revealed an anechoic cystic structure in the irregularly thickened stomach wall in the greater curvature of the antrum.

Laboratory diagnosis

Complete blood count, serum tumor markers (carcinoembryonic antigen, alpha-

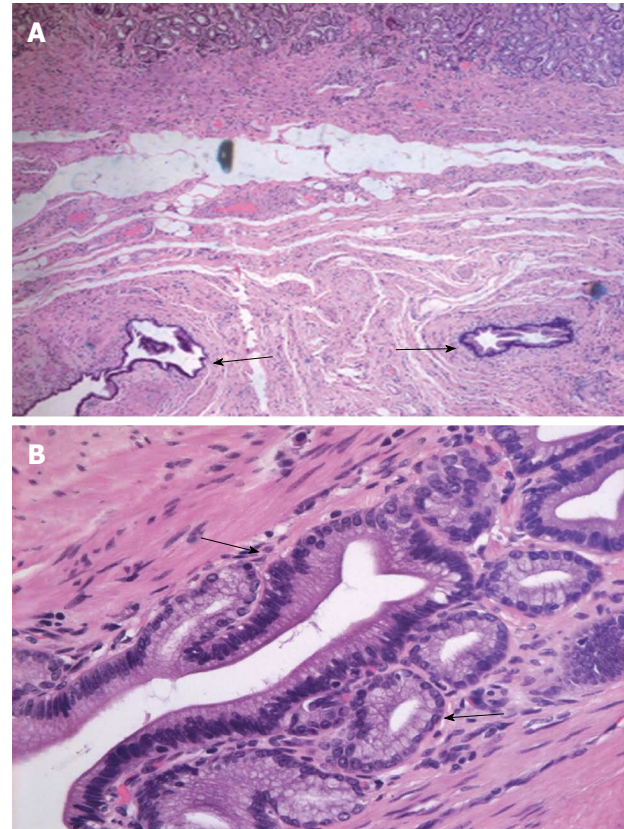


Figure 4 Histology. Hematoxylin and eosin staining of nodular lesion specimens showed dilated cystic glands (arrows) in the A: Muscularis mucosa ($\times 50$ magnification); B: Submucosal layers ($\times 400$ magnification).

fetoprotein, carbohydrate antigen 19-9), metabolic panel and liver function test were within normal limits.

Imaging diagnosis

Abdominal computed tomography and magnetic resonance imaging showed the gastric wall on the greater curvature of the antrum was markedly and irregularly thickened; enhanced scanning showed mild to moderate enhancement of the lesion periphery, with no enhancement in the central portion of the lesion.

Pathological diagnosis

Postoperative pathologic examination showed a 2.5 cm \times 1.5 cm lesion with an eroded mucosal surface, confirming the diagnosis of gastritis cystica profunda (GCP) without malignancy.

Treatment

The patient underwent a distal gastric resection (Billroth I).

Related reports

GCP usually emerges as giant gastric folds, submucosal tumors, or isolated polyps at the anastomotic sites of previous surgeries. GCP is also more prevalent in patients with gastric cancer, and can be considered as a precancerous lesion.

Term explanation

GCP is a rare disease characterized by polypoid hyperplasia and cystic dilatation of the gastric glands extending into the submucosa of the stomach.

Experiences and lessons

Although GCP is typically only seen in stomachs of patients who have undergone gastric procedures, it can also develop in stomachs of patients with no history of abdominal surgery.

Peer-review

In this article, the authors report a rare case of GCP that developed in that stomach of patient who had not previously undergone gastric surgery. The authors also review the clinical pathologic features of GCP. It is a case report that is of interest to the readership.

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