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Hepatitis C virus genotypes in Myanmar

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Abstract

Myanmar is adjacent to India, Bangladesh, Thailand, Laos and China. In Myanmar, the prevalence of hepatitis C virus (HCV) infection is 2%, and HCV infection accounts for 25% of hepatocellular carcinoma. In this study, we reviewed the prevalence of HCV genotypes in Myanmar. HCV genotypes 1, 3 and 6 were observed in volunteer blood donors in and around the Myanmar city of Yangon. Although there are several reports of HCV genotype 6 and its variants in Myanmar, the distribution of the HCV genotypes has not been well documented in areas other than Yangon. Previous studies showed that treatment with peginterferon and a weight-based dose of ribavirin for 24 or 48 wk could lead to an 80%-100% sustained virological response (SVR) rates in Myanmar. Current interferon-free treatments could lead to higher SVR rates (90%-95%) in patients infected with almost all HCV genotypes other than HCV genotype 3. In an era of heavy reliance on direct-acting antivirals against HCV, there is an increasing need to measure HCV genotypes, and this need will also increase specifically in Myanmar. Current available information of HCV genotypes were mostly from Yangon and other countries than Myanmar. The prevalence of HCV genotypes in Myanmar should be determined.

Key words: Direct-acting antivirals; Genotypes; Hepatitis C virus; Interferon-free; Myanmar

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Core tip: We reviewed the prevalence of hepatitis C virus (HCV) genotypes in Myanmar. HCV genotypes 1, 3 and 6 were observed in volunteer blood donors in and around the Myanmar city of Yangon. Although there are several reports of HCV genotype 6 in Myanmar, the distribution of HCV genotypes has not been well documented in areas other than Yangon. Previous studies showed that treatment with peginterferon and a weight-based dose of ribavirin for 24 or 48 wk could

lead to an 80%-100% sustained virological response in Myanmar.

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INTRODUCTION

The hepatitis C virus (HCV) is a single and positive-stranded RNA virus that is approximately 9600 nucleotides in length^[1,2]. HCV infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)^[3]. HCV is classified into at least 7 confirmed genotypes and 67 subtypes^[4].

Myanmar is located in Southeast Asia and has recently emerged as a nation that is pursuing a peaceful transition to democracy^[5]. In Myanmar, the prevalence of HCV infection is 2%, and it accounts for 25% of HCC^[5]. HCV genotypes influence the outcome and the duration of interferon-based^[6,7] and interferon-free treatments^[8]. Direct-acting antivirals (DAAs) against HCV could lead to higher sustained virological response (SVR) rates with fewer adverse events^[9,10]. HCV genotypes are very important factors in the selection of DAAs and treatment regimens^[9,10].

Myanmar is adjacent to India, Bangladesh, Thailand, Laos and China. In India, HCV genotypes 3a, 3b, 1b and 1a have prevalence rates of 50%, 25%, 14% and 10%, respectively, whereas HCV genotype 4 has only a 4% prevalence rate and has been found only in Southern and Western India^[11]. The most common HCV genotypes in patients with chronic HCV infection in Bangladesh were types 3 (50%), 3 and 4 (29%), and 1 (14.4%)^[12]. In blood donors in Thailand, the most common HCV genotype was 3a (43%), followed by 1b (13%), 6f (13%), 6i (8.7%), 1a (4.4%), 3b (4.4%), 6c (4.4%), 6j (4.4%), and 6n (4.4%)^[13]. In Laos, HCV genotypes 1 and 6 have prevalence rates of 5% and 95%, respectively^[14]. In Mainland China, 42%, 44% and 14% of patients have HCV genotypes 1, 2 and 3, respectively^[15]. In this article, we reviewed the prevalence of HCV genotypes in Myanmar.

HCV GENOTYPES IN MYANMAR

Distribution of HCV genotypes in Myanmar

Nakai *et al.*^[16] reported that the most common HCV genotype among 24 patients with liver diseases who were examined at Yangon General Hospital, Yangon, Myanmar, was 3b (67%), followed by genotypes 1a (13%) and 3a (8%); the genotypes were determined using PCR with genotype-specific primers. Mellor *et al.*^[17] used the PCR-restriction fragment length polymorphism

method to show that variants of HCV genotype 6 exist in Myanmar. Shinji *et al.*^[18] reported prevalence rates of 31%, 47% and 21% for variants of HCV genotypes 1, 3 and 6 variants, respectively, in volunteer blood donors in and around the Myanmar city of Yangon; these findings were determined using direct sequencing of PCR products. Previous reports^[16-19] showed the inconsistent data of the prevalence of HCV genotypes among the different area in Myanmar (Table 1), suggesting that this difference may attribute to the regional difference (Figure 1).

HCV genotype 6 in Myanmar

Lwin *et al.*^[19] reported that HCV genotype 6 was the most prevalent genotype (49%), followed by HCV genotypes 3 (39%), 1 (11%), and 2 (0.7%). In Myanmar, HCV genotype 6 was most often found in patients in the northern cities and HCV genotype 3 in the southern and western cities, suggesting that there are regional differences in HCV genotype distribution^[19].

In the Yunnan province in China, where is located in the far southern part of Mainland China bordering Laos, Vietnam, and Myanmar, HCV genotypes 1a, 1b, 3a, 3b, 6a, 6n, and 6u were found in 1.3%, 20%, 24%, 30%, 5%, 11% and 8.8%, respectively, of patients who were co-infected with HCV and HIV^[20,21]. A similar HCV genotype distribution of intravenous drug users was reported in this area^[22]. Zhang *et al.*^[22] reported that HCV genotype 6 was most common (47%), followed by HCV genotypes 3 (41%) and 1 (12%) in intravenous drug users of the Yunnan province. Lwin *et al.*^[19] reported that HCV genotypes 1a, 3a, 3b, and 6 were found in 9%, 11%, 20%, and 60%, respectively, of patients in Muse where is located adjacent to the Yunnan. There seems to be the some association of HCV genotype distribution between Muse in Myanmar and Yunnan province in Mainland China.

In a large number of immigrant workers from Cambodia and Myanmar to Thailand, the predominant HCV genotypes were 1a, 1b, 3a, 3b and 6 (6e, 6f, 6m, 6p and 6r)^[23]. The seroprevalence of HCV infection in immigrant workers from Cambodia and Myanmar to Thailand was reported to be 2.3% or 1.7%, respectively. HCV genotypes 1a, 1b, 3a, 3b and 6 were 0%, 24%, 16%, 4% and 56%, respectively, in immigrant workers from Cambodia to Thailand, and those were 6.7%, 6.7%, 26.7%, 33.3% and 26.6%, respectively, in immigrant workers from Myanmar to Thailand. Geographic distribution of HCV genotype 6 covered mainly southern China and the mainland of Southeast Asia, including Vietnam, Laos, Thailand, Cambodia and Myanmar^[24-27].

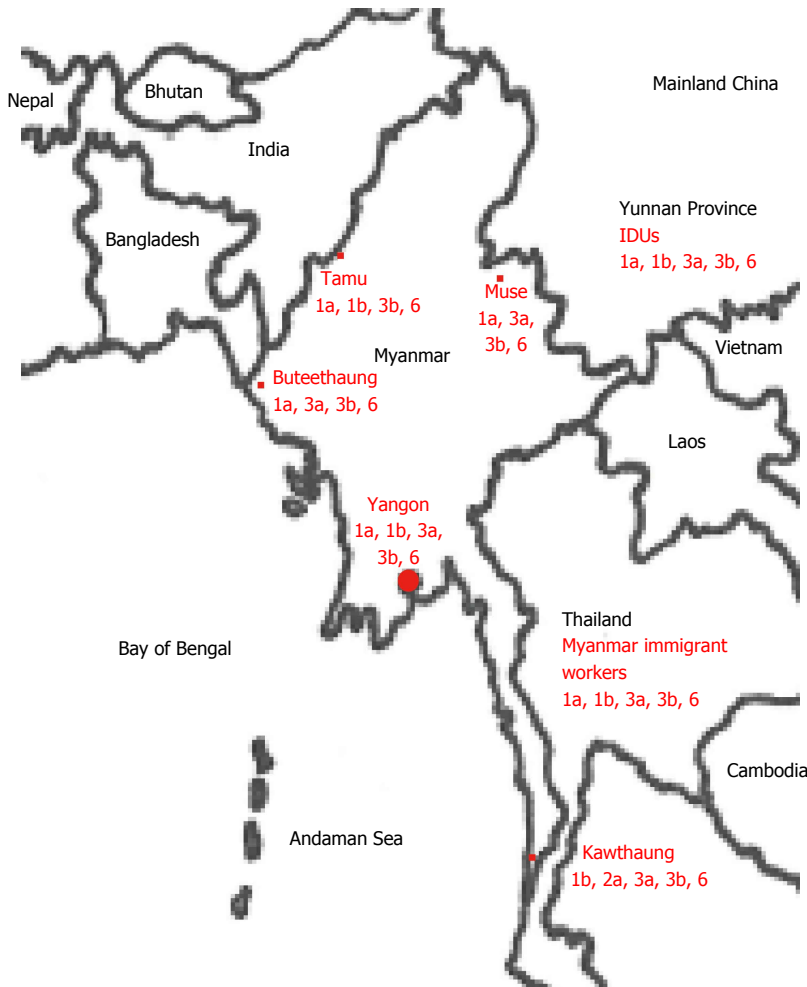
RESPONSE TO PEGINTERFERON AND RIBAVIRIN

Treatment of peginterferon and a weight-based dose of

Table 1 Hepatitis C virus genotypes in the different regions of Myanmar

Ref.	Area/subjects	GT-1a	GT-1b	GT-2a	GT-3a	GT-3b	GT-6
Nakai <i>et al</i> ^[16] , 2001	Yangon/liver diseases	13%	4%	-	8%	67%	-
Shinji <i>et al</i> ^[18] , 2004	Yangon/healthy blood donors	4.5%	20%	-	12%	32%	21%
Lwin <i>et al</i> ^[19] , 2007	Muse/healthy people	9%	-	-	11%	20%	60%
Lwin <i>et al</i> ^[19] , 2007	Tamu/healthy people	2%	15%	-	-	25%	58%
Lwin <i>et al</i> ^[19] , 2007	Kawthaung/healthy people	-	7%	7%	13%	33%	40%
Lwin <i>et al</i> ^[19] , 2007	Buteethaung/healthy people	4%	-	-	27%	53%	16%
Akkarathamrongsin <i>et al</i> ^[23] , 2011	Thailand/immigrant workers from Myanmar	6.7%	6.7%	-	27%	33%	27%

GT: Genotype; -: None.

**Figure 1** Distribution of hepatitis C virus genotypes in Myanmar^[18,19,22,23]. IDUs: Intravenous drug users.

ribavirin for 24 or 48 wk were given to patients infected with HCV genotypes 2 and 3 or HCV genotypes 1 and 6. SVR rates were 81.2% (39/48), 100% (2/2), 85.5% (94/110), 90.3% (28/31) and 100% (4/4) in patients infected with HCV genotypes 1, 2, 3, 6 and with an indeterminate genotype, respectively^[5].

CONCLUSION

DAA is currently available and will be available in the near future to treat patients infected with HCV. In regards to the use of DAAs, the importance of

measuring HCV genotypes is increasing and will also increase specifically in Myanmar. DAAs against HCV could lead to higher SVR rates (90%-95%) in patients infected with almost all HCV genotypes other than HCV genotype 3^[9,10,28]. With current DAAs, HCV genotype 3 is the most difficult-to cure HCV genotype^[28]. HCV NS5B polymerase nucleotide inhibitor sofosbuvir plus ribavirin for 12 and 24 wk could lead 61%-68% and 94% SVR rates, respectively, in non-cirrhotic treatment-naïve patients with HCV genotype 3^[28]. Those could lead only 21%-34% and 92% SVR rates, respectively, in cirrhotic treatment-naïve patients with

HCV genotype 3^[28]. In treatment-experienced patients, those treatments could lead to less SVR rates^[28]. In patients with HCV genotype 6, HCV NS5A inhibitor ledipasvir plus sofosbuvir could lead to approximately 96% SVR rates^[28]. Most of the data of HCV genotypes were from Yangon and countries other than Myanmar. It is important to determine the prevalence of HCV genotypes in Myanmar.

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2016 Hepatocellular Carcinoma: Global view

Diabetes mellitus and metformin in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the leading cause of cancer-related death worldwide. Diabetes mellitus, a risk factor for cancer, is also globally endemic. The clinical link between these two diseases has been the subject of investigation for a century, and diabetes mellitus has been established as a risk factor for HCC. Accordingly, metformin, a first-line oral anti-diabetic, was first proposed as a candidate anti-cancer agent in 2005 in a cohort study in Scotland. Several subsequent large cohort studies and randomized controlled trials have not demonstrated significant efficacy for metformin in suppressing HCC incidence and mortality in diabetic patients; however, two recent randomized controlled trials have reported positive data for the tumor-preventive potential of metformin in non-diabetic subjects. The search for biological links between cancer and diabetes has revealed intracellular pathways that are shared by cancer and diabetes. The signal transduction mechanisms by which metformin suppresses carcinogenesis in cell lines or xenograft tissues and improves chemoresistance in cancer stem cells have also been elucidated. This review addresses the clinical and biological links between HCC and diabetes mellitus and the anti-cancer activity of metformin in clinical studies and basic experiments.

Key words: Hepatocellular carcinoma; Diabetes mellitus; Metformin; Risk

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Core tip: Diabetes mellitus, an increasing risk factor for hepatocellular carcinoma (HCC), shares pathological mechanisms with HCC. Thus, the first-line anti-diabetic metformin was anticipated to reduce cancer risk.

Though basic research has provided evidence of its anti-cancer effect, clinical studies of diabetic patients have not provided conclusive data that metformin reduces HCC risk. Clinical studies have suggested that metformin may suppress cancer in non-diabetic subjects. Basic research on cancer stem cell-targeting therapies has also examined the potential of metformin.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most frequent cancer worldwide and the third most common cause of cancer death^[1,2]. This primary liver cancer originating from hepatocytes is characterized by poor prognosis, and patients with HCC incident risks must be monitored closely for HCC occurrence^[3]. Patients in the early stage of malignancy onset can be cured by surgery^[4-6] or radiofrequency ablation therapy^[7,8]. However, HCC recurs in 60%-80% of patients within 5 years of curative treatments^[9-12] due to intrahepatic metastasis or multicentric occurrence facilitated by the long-term exposure of the liver to chronic viral hepatitis, fibrotic changes and hyperinsulinemia. Patients who do not meet the criteria for surgical resection or radiofrequency ablation therapy can be treated with either transcatheter arterial chemoembolization therapy^[13,14], an anti-cancer drug and lipiodol emulsion^[15], or drug-eluting beads^[16] with the intention of downstaging the tumor to enable hepatic resection or await liver transplantation. Liver transplantation, a radical curative surgery with a 3-year overall survival rate of approximately 40%, has been established as a standard treatment for HCC using the Milan criteria^[17]. However, patients who require liver transplantation must confront a shortage of donors and progression of their cancer stage while awaiting a donor, particularly in Asia^[18]. Patients who fail or are excluded from any of the above treatments might receive sorafenib, a multi-kinase inhibitor that has exhibited effectiveness for improving HCC patient prognosis in randomized control trials^[19,20] but improves survival by only 3 mo.

Therefore, the prevention of HCC and the intense screening of high-risk individuals are of great importance. The risk factors for HCC are now well-established, and great efforts have been made to decrease HCC prevalence, recurrence and death. The medical histories of HCC patients range from liver-specific viral infections, such as HBV and HCV, and alcohol consumption to metabolic disorders including diabetes mellitus and obesity^[21]. Effective anti-viral

agents for HBV and HCV significantly suppressed HCC prevalence in clinical trials^[22-25]. However, type 2 diabetes mellitus, characterized by hyperinsulinemia in its early stage and usually linked to obesity, is increasing worldwide.

Metformin, an oral anti-diabetic drug that is less expensive than any other anti-cancer agent in use, first attracted attention in 2005 for its potential to suppress not only serum glucose levels but also the incidence of various cancers in an observational study^[26]. Metformin has subsequently been investigated as an anti-cancer medicine for malignancies including HCC in diabetic and non-diabetic subjects. This review describes the links between HCC and type 2 diabetes mellitus in terms of their epidemiology and pathology and addresses the benefits and limitations of metformin in the prevention and treatment of HCC.

DIABETES MELLITUS IN HCC

EPIDEMIOLOGICAL TRENDS

Some trends of HCC epidemiology have recently attracted attention. First, the increase in HCC incidence rates has ceased in two nations, Japan and the United States^[27-29], though the prevalence of HCC and HCC-related deaths will continue to increase in the future^[2,30]. The restriction of these phenomena to these two developed countries reflects the diversity of HCC incidence with geography, socioeconomic condition, race, generation and gender in these two countries^[31]. In Japan, liver cancer incidence rates rapidly increased between the mid-1970s and 1990s but then leveled off and began to decrease by 2003^[27,32]. This trend in a Japanese city has been attributed to a decline in HCV infection, previously the major cause of HCC in Japan, partly by avoiding HCV-contaminated blood transfusion and suppression and by restraining injections of drugs of abuse after the Second World War. In the United States, the absence of a significant increase in HCC incidence rates is due in part to the decreased prevalence of HCC among the largest group with an HCC risk caused by HBV, Asians/Pacific islanders, particularly men aged 35-49 years^[28].

Second, a frequent risk factor for HCC, the hepatitis C virus, can now be more easily and thoroughly eradicated by direct anti-viral agents^[24,25], which are newly developed oral medications that might affect HCC prevalence more strongly than interferon^[33,34]. HBV is another hepatocarcinogenic virus that infects hundreds of millions of people globally. The replication and inflammatory activity of HBV in a HBV-infected liver can be inhibited by the newly developed nucleotide analogues entecavir^[22,23] and tenofovir^[35]. These two retrograde transcriptional viral inhibitors have largely overcome the disadvantages of the previous nucleotide analogues, lamivudine and adefovir, which usually caused viral mutations and drug resistance in 70% of patients who took lamivudine for

4 years^[36] and in 20% of patients who were prescribed adefovir for 5 years^[37,38].

Third, the prevalence of another HCC risk factor, type 2 diabetes mellitus, continues to increase^[39,40] with the increase in the incidence of obesity^[41]. The number of diabetic patients is estimated to increase to 300-400 million worldwide by 2030^[39,40,42]. Such predictions are based on increases in populations living in urban areas in developing countries, in senior diabetic patients and in obesity.

DIABETES MELLITUS AS A RISK FACTOR FOR HCC

Diabetes mellitus was first investigated as a risk factor for cancer death at the beginning of the 20th century, when the etiologies of these two major deadly diseases were unknown. An observational study addressing cancer deaths in United States cities in 1910 concluded that the correlation between cancer and diabetes mellitus was not fortuitous nor due merely to errors of observation^[43], although cancer prevalence at that time was biased by the availability of medical schools at which post-mortem examinations and cancer diagnoses could be performed, as discussed by Greenwood^[44]. Greenwood himself analyzed the correlation between death rates due to diabetes and cancer. He concluded that cancer mortality was significantly associated with diabetes in the United States and not in Europe, but he did not assess the correlation between organ-specific cancer deaths and diabetes^[44].

Among various organ-specific cancers, pancreatic cancer was initially determined to have coincidence with diabetic conditions in an observation of 10000 diabetic patients in 1934, although that conclusion may have been due to reverse causation^[45]. In 1970, Kessler reported an association between pancreatic cancer mortality and diabetes mellitus prevalence in human males, although no excess deaths *via* any other type of cancer were observed among diabetic patients; this phenomenon occurred in part because diabetic patients died from diabetes itself or from cardiovascular diseases before cancers other than those of pancreatic origin became fatal^[46].

The strength of the association between cancers and diabetes depends on the cancer species; however, a hospital-based case control study in 1986 demonstrated that more than 4 times as many HCC patients suffered from diabetes mellitus than colorectal tumor patients and femoral bone fracture patients^[47]. A large population-based cohort study in Uppsala, Sweden, confirmed the significantly increased risk of HCC as well as pancreatic cancer in diabetic patients with a relative ratio of approximately 1.5, which was higher in males than in females^[48]. The link between diabetes mellitus and the larger HCC population is supported by two major prospective cohort studies, one in Sweden^[49] and the other in Denmark^[50],

followed by another case-control study in Italy^[51]. Diabetes mellitus has subsequently been investigated as a risk factor for the prevalence^[52,53], recurrence^[54-57] and mortality^[58,59] of HCC.

Diabetes mellitus is now considered an independent risk factor for HCC^[60,61] and has been proven to increase the risk of HCC even in those not infected with HBV or HCV^[57,62,63]. Increased incidence and mortality of several malignancies other than HCC, including pancreatic cancer, endometrial cancer and colon cancer, have been observed among diabetic patients in a series of studies^[48,58,64], partly based on obesity, which has also been identified as a risk factor for cancers including HCC^[65,66]. An association between post-load plasma glucose in a non-diabetic individual who has the potential to develop diabetes mellitus and cancer mortality due to HCC has also been suggested^[67,68].

These observational studies assessing the potential role of diabetes mellitus as a risk factor for cancers were not free from detection bias and reverse causation; the cancer risk was highest immediately after the diabetes cases were registered or diagnosed in each study and then decreased gradually with time^[69]. However, the risks of HCC, pancreatic cancer and endometrial cancer remained significant after adjusting for detection bias and reverse causation^[70]. Major clinical studies of the relationships between diabetes mellitus and HCC and other cancers are illustrated in Table 1.

BIOLOGICAL LINKAGE BETWEEN DIABETES MELLITUS AND CANCER

In 1910, Maynard hypothesized that cancer occurrence might be due to meteorological conditions, such as hours of sunshine, mean temperature, rainfall and other indicators, but observed no significant correlations^[43]. He subsequently focused on diabetes mellitus as a possible cause of cancer because the two diseases occurred at similar ages, were increasing in prevalence and had no known etiologies at that time, as discussed by Greenwood^[44]. The subsequent body of research has since established that the etiologies of diabetes mellitus and cancer share a number of biological pathways^[61], some of which are based on central obesity and insulin resistance, common risk factors for both diseases^[71].

The insulin/IGF-1 axis involves over-activation of mTOR

The classical pathways shared by diabetes mellitus and cancer are the Insulin/IGF- axis, including over-activation of mTOR. Type 2 diabetes mellitus is characterized by hyperinsulinemia. Insulin exerts its proliferative effects directly through the insulin receptor (IR) and indirectly by increasing circulating levels of IGF-1^[72]. Insulin increases circulating IGF-1 by decreasing hepatic production of IGF-binding protein

Table 1 The influence of diabetes mellitus on the incidence, recurrence, and mortality of hepatocellular carcinoma

Ref.	Year	Study design	Type of diabetes	Results
Maynard ^[43]	1910	Case-control	Not differentiated	Cancer mortality increased
Greenwood and Wood ^[44]	1914	Case-control	Not differentiated	Cancer mortality increased in American cities; no significant correlation was observed in European cities
Marble ^[45]	1934	Case-control	Not differentiated	Pancreatic cancer incidence increased
Kessler ^[46]	1970	Case-control	Not differentiated	Pancreatic cancer deaths increased
Lawson <i>et al</i> ^[47]	1986	Case-control	Not differentiated	HCC incidence increased (HR = 3.9)
Levine <i>et al</i> ^[67]	1990	Cohort	IGT	HCC deaths increased in men; post-load plasma glucose increased
Adami <i>et al</i> ^[48]	1991	Cohort	Not differentiated	Incidences of primary liver (RR = 1.5), pancreatic (RR = 1.4) and endometrial (RR = 1.5) cancers increased
Smith <i>et al</i> ^[164]	1992	Cohort	IGT	Pancreatic cancer increased (RR = 2.25); post-load plasma glucose increased in IGT men. HCC was not analyzed in organ-specific statistics
La Vecchia <i>et al</i> ^[51]	1994	Case-control	Not differentiated	Liver cancer incidence remained elevated 10 yr after the diagnosis of diabetes (RR = 2.6)
Adami <i>et al</i> ^[49]	1996	Cohort	Not differentiated	Primary liver cancer incidence increased (SIR = 4.7 in men and 3.4 in women)
Wideroff <i>et al</i> ^[50]	1997	Cohort	Not differentiated	Primary liver cancer incidence increased (SIR = 4.0 in men and 2.1 in women)
La Vecchia <i>et al</i> ^[165]	1997	Case-control	Not differentiated	Liver cancer incidence increased (OR = 2.2) for at least 10 yr after the diagnosis of diabetes
Ikeda <i>et al</i> ^[54]	1998	Cohort	Not differentiated	Recurrence-free survival after hepatic resection decreased in diabetic cases
Balkau <i>et al</i> ^[52]	2001	Cohort	Not differentiated	HCC incidence increased with fasting hyperinsulinemia (HR = 2.72) and 2-h hyperinsulinemia (HR = 3.41)
Huo <i>et al</i> ^[55]	2003	Cohort	Not differentiated	HCC recurrence increased in HBV-seropositive cases
Coughlin <i>et al</i> ^[58]	2004	Cohort	Not differentiated	Liver cancer mortality increased in men (RR = 2.19)
Batty <i>et al</i> ^[68]	2004	Cohort	IGT	HCC (HR = 2.47) and pancreatic cancer (HR = 1.35) increased; post-load plasma glucose increased in IGT men
El-Serag <i>et al</i> ^[60]	2006	Meta-analysis	Not differentiated	HCC incidence increased in 9 case-control studies (OR = 2.5) and 7 cohort studies (OR = 2.5)
Inoue <i>et al</i> ^[64]	2006	Cohort	Not differentiated	HCC incidence increased (HR = 2.24 in men and 1.94 in women)
Komura <i>et al</i> ^[56]	2007	Cohort	Not differentiated	Postoperative recurrence-free survival decreased in diabetic cases
Kawamura <i>et al</i> ^[57]	2008	Cohort	Not differentiated	HCC recurrence increased (HR = 4.61)
Landman <i>et al</i> ^[59]	2010	Cohort	Type 2	HCC death increased (SMR = 1.47)
Lee <i>et al</i> ^[53]	2011	Cohort	Type 2	Incidences of total cancer, HCC and pancreatic cancer increased
Hense <i>et al</i> ^[166]	2011	Cohort	Type 2	HCC incidence increased (SIR = 1.94)
Johnson <i>et al</i> ^[70]	2011	Cohort	Type 2	After detection biases were excluded, incidences of HCC (HR = 2.53), pancreatic (HR = 1.65) and endometrial (HR = 1.58) cancers increased
Wang <i>et al</i> ^[167]	2012	Meta-analysis	Type 1 and type 2	HCC incidence (RR = 2.23) and mortality (RR = 2.43) increased in cohort studies
Wang <i>et al</i> ^[168]	2012	Meta-analysis	Not differentiated	HCC incidence (RR = 2.01) and mortality (RR = 1.56) increased
Lai <i>et al</i> ^[101]	2012	Cohort	Not differentiated	HCC incidence increased (HR = 1.73)
Schlesinger <i>et al</i> ^[62]	2013	Cohort	Not differentiated	HCC incidence increased (RR = 2.17) in HBV/HCV-negative individuals
Koh <i>et al</i> ^[63]	2013	Cohort	Not differentiated	HCC incidence increased (HR = 2.14), particularly in non-viral cases (HR = 5.15)
Wang <i>et al</i> ^[169]	2014	Meta-analysis	Not differentiated	HCC in diabetic cases was related to overall survival (RR = 1.46) and disease-free survival (RR = 1.57)
Harding <i>et al</i> ^[69]	2015	Case-control	Type 1 and type 2	Incidences of total, liver, pancreatic and endometrial cancer increased in cases involving type 2 diabetes mellitus

RR: Relative risk; OR: Odds ratio; HR: Hazard ratio; SIR: Standardized incidence ratio; SMR: Standardized mortality ratio; IGT: Impaired glucose tolerance.

1, a ligand of IGF-1, thus increasing levels of free IGF-1^[73,74]. In hyperinsulinemia, the activity of insulin becomes less metabolic and more mitogenic. Insulin decreases its metabolic activity by over-activation of mTOR, which phosphorylates IR-substrate-1 and attenuates metabolic pathways downstream of insulin signals. Simultaneously, insulin up-regulates IR-substrate-2 and induces the mitogen-activated kinase pathway, thereby enhancing cell survival^[75].

Hyperinsulinemia is regarded as an independent risk factor for HCC, and major dysregulations of insulin-

dependent pathways have been reported in HCC^[76]. The effect of HCC on development also depends on excess signals from IGF- I but more strongly on signals from IGF- II^[77]. Aberrant mTOR signaling in HCC has been confirmed in human tumor samples^[78].

Chronic inflammation caused by adipokines

Central obesity, which usually accompanies type 2 diabetes mellitus, may be a trigger for carcinogenesis *via* pro-inflammatory cytokines secreted from visceral adipose tissues. Adipokines such as tumor necrosis

factor- α and interleukin-6 are produced in the excess visceral fatty compartment and perpetuate chronic low-grade inflammation in peripheral tissues, which provides microenvironments suitable for tumorigenesis^[79]. Another adipose tissue-derived hormone, leptin, promotes or suppresses cell proliferation^[80]. Adiponectin, which is produced most highly by adipokines, presents both anti-inflammatory and anti-tumor activities^[81].

However, in the case of HCC, the carcinogenic or anti-tumor effects of the two hormones, leptin and adiponectin, have been the subject of contradictory reports, and more conclusive data are needed^[82].

Hyperglycemia

Hyperglycemia is a feature of diabetes mellitus, and Warburg first hypothesized that hyperglycemia itself might have carcinogenic potential^[83]. In general, cancer cells produce ATP by anaerophilic glycolysis. Under aerophilic conditions, cytoplasmic ATP production *via* glycolysis is less efficient than synthesis *via* oxidative phosphorylation in mitochondria. Cancer cells are consequently assumed to require more glucose than normal cells, and several types of cancers have been detected by positron emission tomography based on this theory^[84].

Direct carcinogenic effects of hyperglycemia combined with the Wnt signaling pathway were recently proposed to promote carcinogenesis, resulting in nuclear beta-catenin accumulation^[85] *via* aberrant acetylation of beta-catenins. A national cohort study in Taiwan revealed a linear relationship between HCC occurrence and HbA1c in hyperglycemia^[86]. In addition, a case-control study in Japan demonstrated that post-challenge hyperglycemia was an independent risk factor for HCC^[87].

However, hyperglycemia has been considered subordinate to hyperinsulinemia as a carcinogen, and a meta-analysis of large randomized controlled trials did not indicate definitive cancer risk reduction by intensive glycaemic controls in patients with type 2 diabetes mellitus^[88,89].

Estrogen

Estrogen is produced primarily in the body fat of postmenopausal women and obesity, a background metabolic disorder of diabetes mellitus, is linked to elevated serum estrogen levels. Therefore, estrogen is recognized as a carcinogenic risk factor for breast, endometrial and ovarian cancers in post-menopausal women^[90].

In HCC, however, primary liver malignancies occur predominantly in males, and male HCC patients usually present with a poorer prognosis than female HCC patients^[91]. Among women, post-menopausal women suffer from an elevated incidence of HCC, which is epidemiologically suppressed by estrogen therapy^[92]. The relatively lower estrogen levels in males compared

to females and in post-menopausal women compared to estrogen-supplemented women suggests that low estrogen might contribute to the more frequent cancer prevalence in men, particularly for HCC, because estrogen appears to suppress HCC development by inactivating chronic low-grade inflammation in the liver^[93].

METFORMIN, A DRUG TO POTENTIALLY PREVENT HCC OCCURRENCE, RECURRENCE AND DEATH

Metformin, a first-line oral anti-diabetic^[94], was associated with reduced prevalence of cancers in type 2 diabetic patients by Evans in 2005^[26]. This pilot case-control study, which did not include site-specific cancer data, provided the foundation for epidemiological studies on the anti-tumor effects of metformin. As a matter of fact, an herb called Galega Officinalis, which contains large amounts of guanidine, the original molecule of metformin, was prescribed as long ago as the 17th century to relieve diabetic symptoms^[95].

A population-based cohort study by Bowker *et al.*^[96] in 2006 demonstrated that cancer mortality in the type 2 diabetic group decreased when metformin was prescribed compared to insulin injection. A Scottish cohort study also demonstrated a protective effect of metformin against total cancers^[97].

In the case of HCC, a case-control study suggested that HCC risk was reduced in male type 2 diabetic patients prescribed metformin^[98] and a subsequent cohort study including male and female subjects^[99]. A hospital-based case-control study in the United States also indicated that metformin reduces the incidence of HCC in type 2 diabetic patients^[100]. A prospective cohort study in Taiwan performed by Lee *et al.*^[53] identified benefits of metformin for HCC prevention compared to other anti-diabetics, with a reduced risk of other tumors, pancreatic and colorectal cancer as well. Another cohort study in Taiwan also demonstrated that the development of HCC was suppressed by metformin administration^[101].

However, observational studies of cancer incidence and mortality are subject to analyses of time-related biases, which have led to debate and controversy^[102,103]. Two retrospective cohort studies observed no influence of metformin on cancer risk^[104,105]. A meta-analysis excluding studies with time-related biases stated that metformin did not significantly reduce the risk of HCC. However, colon cancer was the only type of cancer that remained significant in a site-specific cancer risk analysis, and a 10% risk reduction for total cancers remained^[106]. A randomized controlled trial comparing metformin to rosiglitazone failed to support a significant difference in cancer occurrence between the two oral anti-diabetics^[107]. A meta-analysis of randomized controlled trials concluded that metformin

provided patients with little benefit with respect to overall mortality compared to other anti-diabetics or insulin therapy and a 10% reduction in mortality compared to placebo or usual care that did not reach statistical significance^[108].

In summary, the limited epidemiological research on the anti-cancer activity of metformin in diabetic patients indicates that this drug definitely exhibited an association with decreased cancer prevalence in case-control studies and cohort studies but has failed all randomized controlled trials in diabetic subjects^[109,110]. No conclusive data from clinical trials regarding the prevention of cancers, including HCC in diabetic subjects, by metformin are available.

Clinical studies of metformin as an adjuvant to conventional chemotherapy and radiotherapy have reported promising data in case-control studies intended for patients with pancreatic cancers^[111], breast cancers^[112], lung cancers^[113] and colorectal cancers^[114]. However, a randomized controlled trial investigating the adjuvant use of metformin with conventional chemotherapy intended for pancreatic cancer failed to demonstrate a significant improvement of overall survival^[115]. As an adjuvant to an estrogen-synthesis inhibitor, metformin is being prescribed to estrogen receptor-positive post-menopausal breast cancer patients without diabetes mellitus in a phase II randomized controlled trial^[116]. No randomized controlled trials are available to demonstrate the feasibility of metformin as an adjuvant to non-surgical therapy.

For HCC, a combination of metformin and radiation therapy yielded prolonged overall survival compared to controls^[117], although an adjuvant to sorafenib resulted in shorter progression-free survival and poorer overall survival^[118]. As for primary cancer prevention, results on the adjuvant use of metformin in clinical trials are not conclusive, and further investigations are needed.

The application of metformin as an agent against premalignant tumor activity is being explored in non-diabetic cases to prevent tumor incidence. Metformin suppressed the incidence of colorectal aberrant crypt foci, surrogate markers of colon cancer, in non-diabetic subjects in a small randomized controlled trial^[119]. A double-blind randomized controlled trial in Japan demonstrated a significant reduction in colorectal polyp formation in non-diabetic patients after 1 year of administration of low-dose metformin^[120]. Similar trials on HCC have not been performed, and the anti-tumor activity of metformin in non-diabetic cases requires further elucidation. The major clinical studies of the anti-cancer effect of metformin against HCC and other cancer types are illustrated in Table 2.

ANTI-CANCER MECHANISM OF METFORMIN

Studies of the anti-cancer mechanism of metformin

have followed basic research on the intracellular signaling downstream of metformin to improve hyperglycemia, hyperlipidemia and hyperinsulinemia. An anti-cancer role of metformin had not been proposed when AMPK was identified as a major target molecule of metformin^[121]. The identification of LKB1, a tumor suppressor gene, upstream of AMPK highlighted the biguanides as candidate anti-cancer drugs^[122,123]. Downstream of AMPK, mTOR, an energy sensor and a gene that plays multiple roles in cell proliferation, was identified^[124,125]. An *in vivo* study employing LKB1 knockout mice clarified that metformin signals in the liver *via* the LKB1/AMPK axis in the context of glucose homeostasis^[126]. A role of the LKB1/AMPK/mTOR axis in carcinogenesis and mediating the anti-cancer signaling of metformin was subsequently identified. Another study in LKB1-AMPK double knockout mice identified an AMPK-independent pathway that improves the diabetic state^[127]. AMPK-independent anti-carcinogenic pathways of metformin have also been investigated.

LKB1/AMPK/mTOR axis

Metformin halts the respiratory chain in mitochondria and increases cell energy stress, which activates LKB1 and AMPK. AMPK activation inhibits mTOR and suppresses cell proliferation^[124,125]. Furthermore, LKB1/AMPK disturbs insulin signals by degrading IR-substrate-1, resulting in the suppression of insulin/IGF-1 signaling^[128], a pathway shared by diabetes and cancer. In lipid metabolism, which is indispensable for tumor growth^[129], metformin directly inhibits fatty acid synthase^[130]. Metformin arrests the cell cycle in malignant cells *via* activated AMPK, which is correlated with the downregulation of cyclin D1 and the upregulation of p21^{CIP} and p27^{KIP}^[131,132].

AMPK-independent pathways

AMPK-independent pathways downstream of metformin vary. Metformin is thought to protect against DNA damage from reactive oxygen species (ROS) by inhibiting ROS production when metformin inhibits the mitochondrial respiratory chain^[133]. Metformin can also bypass AMPK and inhibit mTOR signaling^[134] and induce cell cycle arrest by down-regulating cyclin D1 *via* p53^[135,136]. An AMPK knockdown study demonstrated that metformin up-regulates apoptosis and autophagy *via* a Stat3/Bcl2 pathway^[137]. Metformin decreases glucose uptake into cancer cell decreases *via* a direct allosteric effect on hexokinase II^[138].

MicroRNAs as mediators of the anti-cancer activity of metformin

Metformin exerts its anti-carcinogenic activity by regulating microRNA (miRNA) expression to down-regulate target messenger RNAs. miRNAs are small non-coding RNAs with a length of 20-25 nucleotides. miRNAs can bind to messenger RNAs at their 3'-UTR

Table 2 Efficacy of metformin on the incidence, recurrence and mortality of hepatocellular carcinoma and other tumors

Ref.	Year	Study design	Type of diabetes	Results
Evans <i>et al</i> ^[26]	2005	Case-control	Type 2	HCC incidence decreased (OR = 0.79)
Bowker <i>et al</i> ^[96]	2006	Cohort	Type 2	Mortality was lower among metformin users than among insulin or sulfonylurea users (HR = 0.77)
Libby <i>et al</i> ^[97]	2009	Cohort	Type 2	Total cancer incidence decreased (HR = 0.63)
Donadon <i>et al</i> ^[98]	2009	Case-control	Type 2	HCC incidence was lower among metformin users (OR = 0.33) than among insulin users (OR = 2.99)
Donadon <i>et al</i> ^[99]	2010	Cohort	Type 2	HCC incidence was lower among metformin users (OR = 0.15) than among insulin or sulfonylurea users
Hassan <i>et al</i> ^[100]	2010	Case-control	Not differentiated	HCC incidence decreased (OR = 0.30)
Home <i>et al</i> ^[107]	2010	Randomized controlled trial	Type 2	Total cancer incidence did not decrease compared with rosiglitazone users
Landman <i>et al</i> ^[159]	2010	Cohort	Type 2	HCC deaths decreased (HR = 0.43)
Hosono <i>et al</i> ^[119]	2010	Randomized controlled trial	Non-diabetic	A surrogate marker of colorectal cancer incidence decreased
Ferrara <i>et al</i> ^[104]	2011	Cohort	Not differentiated	No decreases in the incidence of any cancer; no data on HCC were available
Lee <i>et al</i> ^[53]	2011	Cohort	Type 2	Incidences of total cancer (HR = 0.12), HCC (HR = 0.06) and colorectal cancer (HR = 0.36) decreased
Hense <i>et al</i> ^[166]	2011	Cohort	Type 2	HCC incidence did not decrease
Lai <i>et al</i> ^[101]	2012	Cohort	Not differentiated	HCC incidence was decreased by metformin (HR = 0.49) and thiazolidinedione (HR = 0.56)
Ruiter <i>et al</i> ^[170]	2012	Cohort	Not differentiated	Incidences of total cancer (HR = 0.90) and HCC (HR = 0.67) were lower among metformin users than among sulfonylurea users
Stevens <i>et al</i> ^[108]	2012	Meta-analysis	Type 2 and at-risk for diabetes	The summary RR for cancer outcomes was 1.02 across all trials
Thakkar <i>et al</i> ^[109]	2013	Meta-analysis	Type 2	Total cancer incidence decreased in case-control studies (RR = 0.90) and cohort studies (RR = 0.70) but did not significantly decrease in randomized controlled trials
Yin <i>et al</i> ^[110]	2013	Meta-analysis	Type 2	Overall survival (HR = 0.65) and cancer-specific survival (HR = 0.62) for total cancers were better for metformin than for other glucose-lowering medications
Tsilidis <i>et al</i> ^[105]	2014	Cohort	Type 2	Incidences of total cancer and HCC were not significantly lower among metformin users than among sulfonylurea users
Gandini <i>et al</i> ^[106]	2014	Meta-analysis	Not differentiated	After adjusting for time-related biases, total cancer incidence decreased (RR = 0.90), but this decrease became insignificant after adjusting for BMI in addition to time-related biases. Total cancer mortality and HCC incidence did not decrease after adjusting for time-related biases
Higurashi <i>et al</i> ^[120]	2016	Randomized controlled trial	Non-diabetic	Incidences of metachronous colorectal adenomas (HR = 0.60) and total polyps (HR = 0.67) decreased

RR: Relative risk; OR: Odds ratio; HR: Hazard ratio.

and inhibit their translation, that is, they regulate gene expression at the post-transcriptional level and modulate biological processes, such as intracellular metabolism, cell proliferation, differentiation, apoptosis and angiogenesis^[139].

Metformin inhibits the cell cycle of various gastrointestinal tumors, including HCC, by up-regulating the let-7 family *in vitro* and *in vivo*^[140-145]. For HCC in particular, metformin's anti-cancer activities are mediated through let-7c, which targets RAS^[146]; miR-140-5p, which targets TGFR1, FGF9^[147] and DNMT1^[148]; and miR-222, which targets PTEN and p57^[149]. In pancreatic cancer cell lines, metformin suppresses HMGA1, a pseudogene gene highly expressed in cancer cells, by up-regulating miR-26a, which binds to and degrades the HMGA1 messenger RNA^[150]. MiRNAs and their target messenger RNAs in cancers originating from other organs have been well summarized by Pulito^[151].

CANCER STEM CELLS AS A TARGET OF METFORMIN IN ADJUVANT THERAPY

Cancer stem cells (CSCs) or tumor-initiating stem cells are a minor subset of the cancer cell population and have been hypothesized to exist among cancer cells. These cells should self-renew indefinitely to generate cancer clones hierarchically and resist chemotherapy and radiotherapy more strongly than any other cancerous daughter cells^[152,153]. CSCs do not express definitive cell surface markers and have not been well defined due to extensive heterogeneity^[154]. However, using surface markers and the enhanced ALDH1 activity of normal stem cells^[155,156], research on CSCs has developed, and a small subset of cells that might include CSCs has been isolated and subjected to further analysis. In the case of HCC, several cell surface markers, such as CD 133, CD90, CD44, EpCAM, OV6 and SP, have been employed to focus on

specific cells, including hepatic CSCs^[157,158].

Tumors have the potential to be resistant to chemotherapy and radiotherapy, and this potential has been attributed to CSCs^[159]. Targeting CSCs in cancer that is refractory to non-surgical treatments may provide a cure. Metformin as an adjuvant to conventional chemotherapy was determined to be effective against CSCs *in vitro* and *in vivo*^[159]. For hepatic CSCs, metformin administration reduced EpCAM-positive cells, partly depending on the AMPK/mTOR pathway in cell lines and xenograft tumors^[160].

CONCLUSION

Diabetes mellitus is globally endemic and has been established as a risk factor for HCC incidence in a large number of observational studies in which researchers critically analyzed study data and adjusted for as many biases as possible. Thus, future increases in diabetes mellitus will likely result in increases in the incidence of HCC. Metformin, a first-line oral anti-diabetic, has been shown to prevent cancer and reduce cancer mortality among diabetic patients in observational studies. Further investigations, particularly randomized controlled trials involving diabetic and non-diabetic subjects, remain necessary. *In vitro* and *in vivo* experiments have already provided evidence of the anti-tumor activity of metformin. Newly developed topics that are being investigated further include the AMPK-independent pathway represented by the LKB1/AMPK/mTOR axis; miRNAs downstream of this biguanide and their messenger RNAs that are pivotal to cell survival and proliferation; and cancer stem cells in HCC that are nearly completely identified using cell surface markers.

In daily clinical practice, the administration of metformin to cancer patients, including those with HCC, is associated with few complications. The biguanides exhibit good tolerance in diabetic patients, even those suffering from cirrhosis^[161]. Lactic acidosis is not significantly associated with metformin^[162]. Although a recent study proposed that metformin might impair cognitive function^[163], causality between metformin prescription and cognitive impairment, including Alzheimer's dementia, has not been confirmed. In summary, metformin is a safe drug for cancer patients as well as diabetic patients. Further clinical evidence of the anti-cancer activity of metformin would have implications for many patients suffering from cancer with or without diabetes mellitus.

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2016 Hepatocellular Carcinoma: Global view

Hepatocellular carcinoma: Will novel targeted drugs really impact the next future?

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Abstract

Cancer treatment has been revolutionized by the advent

of new molecular targeted and immunotherapeutic agents. Identification of the role of tumor angiogenesis changed the understanding of many tumors. After the unsuccessful results with chemotherapy, sorafenib, by interfering with angiogenic pathways, has become pivotal in the treatment of hepatocellular carcinoma. Sorafenib is the only systemic treatment to show a modest but statistically significant survival benefit. All novel drugs and strategies for treatment of advanced hepatocellular carcinoma must be compared with the results obtained with sorafenib, but no new drug or drug combination has yet achieved better results. In our opinion, the efforts to impact the natural history of the disease will be directed not only to drug development but also to understanding the underlying liver disease (usually hepatitis B virus- or hepatitis C virus-related) and to interrupting the progression of cirrhosis. It will be important to define the role and amount of mutations in the complex pathogenesis of hepatocellular carcinoma and to better integrate locoregional and systemic therapies. It will be important also to optimize the therapeutic strategies with existing chemotherapeutic drugs and new targeted agents.

Key words: Hepatocellular carcinoma; Targeted therapy; Pathway; Angiogenesis; Sorafenib

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Core tip: Hepatocellular carcinoma (HCC) is a tumor with increasing incidence and epidemiologic relevance. Advanced hepatocellular carcinoma that is not amenable to radical treatments (*i.e.*, transplantation or surgical resection) has a dismal prognosis (1-2 mo). Sorafenib, a tyrosine kinase inhibitor which targets multiple pro-angiogenic factors, is a cornerstone in the history of HCC treatments. Since the introduction of sorafenib, novel biological drugs have been investigated in hepatocellular carcinoma patients, but no monotherapy or combination therapy has significantly improved

outcomes in clinical trials. Insights into tumor gene profile are critical in recognizing various classes of hepatocellular carcinoma in order to help determine which therapeutic approaches will be beneficial. Well-designed clinical trials may disclose differences in efficacy end-points, thus leading the way to clinical use.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the second most common cause of cancer-related death^[1]. Without specific treatment, HCC has a very poor prognosis: the median survival for patients with early and advanced tumors is 6-9 mo and 1-2 mo, respectively. The occurrence of HCC is associated mainly with endemic hepatitis B virus (HBV) infection and aflatoxin B1 exposure in Africa and Asia, with hepatitis C virus (HCV) infection and non-alcoholic steatohepatitis, in Western countries and Japan. Increasing attention is being given to the mechanisms underlying the development of HCC. In fact HCV, HBV and non-alcoholic steatohepatitis are the primary determinants of hepatocarcinogenesis, and any pharmacologic intervention, from prevention to antiviral therapies, may significantly impact HCC development and growth and, thereafter, response to anti-cancer treatments^[2]. In addition to tumor progression, functional liver impairment due to cirrhosis influences drug metabolism and, ultimately, the patients' outcome. Regardless of the underlying causes of HCC, most of the morbidity and mortality results from the cirrhosis-related complications: ascites, hepatic encephalopathy, variceal hemorrhage, and hepatorenal syndrome. The unsuccessful medical treatment of HCC is, at least in part, due to complex molecular alterations present in HCC tissue and to the activation of multiple signal transduction pathways that control cell proliferation and tumor progression^[3]. Immune-mediated chronic inflammation in hepatitis promotes progressive fibrosis and development of liver cirrhosis, which themselves are early factors responsible for carcinogenesis^[2,4]. Integration of HBV DNA into the host genome not only induces chromosomal instability but, depending on the site of DNA integration, may activate oncogenes or inactivate tumor-suppressor genes^[4].

The critical signaling pathways for HCC are the Wnt/ β -catenin pathway, chromatin remodeling, oxidative stress and signaling involving vascular endothelial growth factor (VEGF), platelet derived growth factor

(PDGF), epidermal growth factor (EGF), fibroblast derived growth factor (FGF), and insulin growth factor (IGF), and intracellular mediators such as RAS/RAF/MAPK and PI3K/AKT^[5]. In Figure 1, a comprehensive representation of pathways involved in HCC and targeted drugs are shown. HCC is considered a relatively chemorefractory tumor. Moreover, underlying cirrhosis and impaired liver function can affect the schedule of administration and activity of chemotherapeutic agents. Response rates achieved with single agents and combination chemotherapies do not exceed 10%-20% in most studies, and encouraging survival benefit has thus far not been shown.

The concept of targeted therapies has emerged as a promising approach for the medical treatment of various cancers, including HCC^[1,3]. Until now, sorafenib (multi-kinase inhibitor) has been the only systemic therapy with a demonstrated survival benefit in HCC. In the SHARP trial^[6], median overall survival was 10.7 mo in the sorafenib group and 7.9 mo in the placebo group (hazard ratio in the sorafenib group, 0.69; 95%CI: 0.55-0.87, $P < 0.001$). Subsequently, several phase III trials, which included patients with intermediate-stage or advanced-stage HCC, investigated first-line and second-line treatments but failed to detect any significant survival benefits.

In this report, we have searched Medline/PubMed through February 5, 2016 for published studies and clinical trials of HCC treatment, including the main drugs involved in advanced study or under investigation. In particular, we selected drugs with published results and those studied in phase II and III trials. Search for clinical trials was performed on <https://clinicaltrials.gov/ct2/search/advanced>, using the search terms hepatocellular carcinoma and "experimental drug", "open studies", "interventional study", with selection of phase 2 and 3 trials. Finally, we have tried to imagine the future areas of clinical investigation most promising in HCC.

DRUGS TARGETING ANGIOGENESIS

Angiogenesis is one of the prominent features of liver cancer and is also one of the targets of sorafenib, the first approved drug in HCC treatment. Tumor angiogenesis is predominantly promoted by VEGF and PDGF. This latter is also linked to increased metastatic potential of HCC^[7].

New trials have been designed with the aim of improving the results obtained with sorafenib single agent^[8].

Phase III trials are evaluating sorafenib in combination with transarterial chemoembolization (TACE) (Table 1). Sorafenib in combination with chemotherapeutic regimens known to be active in HCC (doxorubicin, FOLFOX or XELOX regimen, 5-fluorouracil/mitomycin) is under evaluation in phase II studies (Table 2). Patients with advanced stages of cirrhosis are usually excluded from clinical studies, so whether and

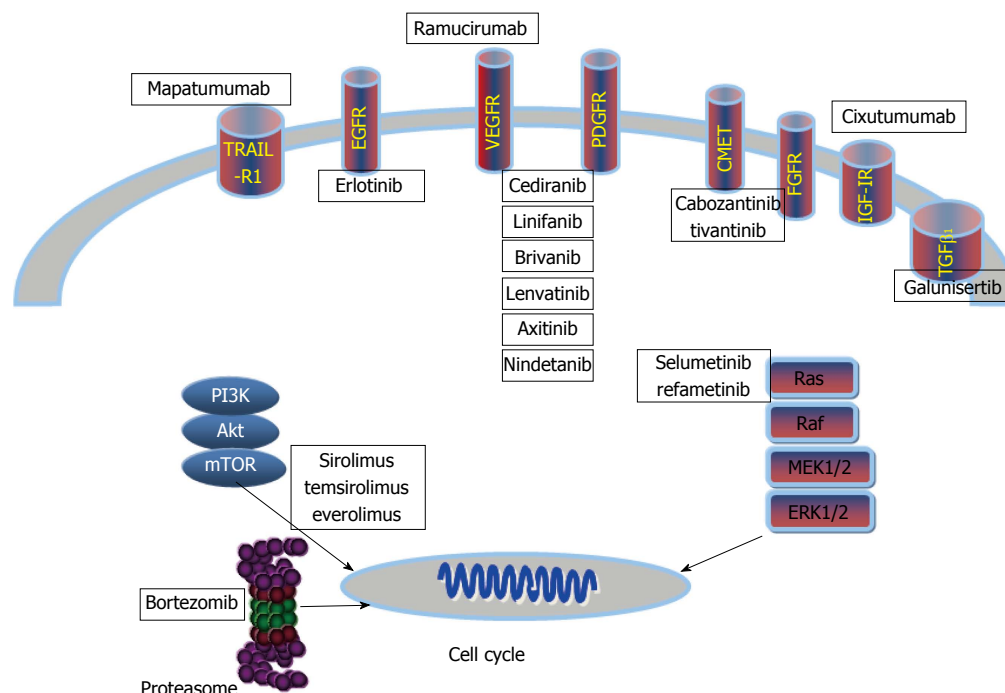


Figure 1 Targeted therapies and signaling pathways in hepatocellular carcinoma.

how to treat these patients is challenging. A Chinese study found similar clinical and progression-free survival benefit among Child-Pugh A and B patients treated with sorafenib^[9]. A retrospective Italian study highlighted the safety of sorafenib across the various Child-Pugh classes^[10]. The Prodigy 21 study is investigating sorafenib in HCC patients with Child B cirrhosis (NCT01357486, Table 2). In that study, two drugs, sorafenib at full doses (400 mg twice a day) and pravastatin, are used in the experimental arms. However, low doses of sorafenib might have clinical activity^[11], as shown preliminarily *in vitro*^[12], and may permit treatment of HCC in patients with advanced Child-Pugh classes who are at increased risk of toxicity. Sorafenib is also under evaluation in combination with stereotactic radiosurgery (RTOG-1112) in early HCC.

Regorafenib is another multi-kinase inhibitor that has growth inhibitory action against a variety of tumors *in vitro*. A phase III trial is testing regorafenib in HCC patients whose disease progressed during sorafenib therapy (NCT01774344; Table 1).

Cediranib (AZD2171) is a potent inhibitor of VEGF receptor tyrosine kinases. Competing with adenosine triphosphate, cediranib binds to and inhibits all three VEGF receptor (VEGF-1,-2,-3) tyrosine kinases, thereby blocking VEGF-signaling, angiogenesis, and tumor-cell growth. Cediranib, 30-mg orally once daily (4 wk/cycle), was tested in a Phase II study, where it resulted in stable disease in 5 of 17 patients (29%), an estimated 3-mo progression-free survival (PFS) rate of 77%, median PFS of 5.3 mo, and a median overall survival of 11.7 mo. In that study, Grade 3 toxicities included hypertension (29%), hyponatremia (29%) and hyperbilirubinemia (18%)^[13]. Despite the authors'

claim of some anti-tumor activity, no further studies are ongoing.

Linifanib (ABT-869) is a novel oral ATP-competitive inhibitor of all VEGF and PDGF receptor tyrosine kinases. Forty-four patients with advanced HCC were treated with 0.25 mg/kg daily. The estimated progression-free rate at 16 wk was 31.8%, the estimated objective response rate (ORR) 9.1%, the median time to progression (TTP) 3.7 mo, and the median overall survival 9.7 mo. The most common adverse events were diarrhea (55%) and fatigue (52%). The most common grade 3/4 adverse events were hypertension (25%) and fatigue (14%)^[14]. A Phase III trial comparing linifanib (17.5 mg daily) and sorafenib in advanced HCC found similar overall survival for the two agents^[15].

Ramucirumab (IMC-1121B) is a fully humanized monoclonal antibody that binds to the extracellular domain of VEGFR-2. The REACH study was a second-line, randomized, placebo-controlled, phase 3 study in patients with advanced HCC after first-line treatment with sorafenib. Median overall survival for the ramucirumab group was 9.2 mo compared with 7.6 mo for the placebo group (HR = 0.87, 95%CI: 0.72-1.05, $P = 0.14$)^[16]. Grade 3 or greater adverse events, occurring in 5% or more of patients in either treatment group, were ascites, hypertension, asthenia, progression of malignant neoplasm, increased aspartate aminotransferase concentration, thrombocytopenia, and increased blood bilirubin values. The authors' conclusion was that second-line treatment with ramucirumab did not significantly improve survival compared with placebo in patients with advanced HCC. A subgroup analysis, conducted to evaluate the relationship between alpha-fetoprotein

Table 1 On-going National Cancer Institute-sponsored phase III trials

Target molecule	Molecule	trial	Phase	Details	Locoregional treatment	Primary outcome	Estimated enrollment	Start date	Estimated study completion date	Ref.
VEGFR	Ramucirumab	Ramucirumab (LY3009806) <i>vs</i> placebo in participants with hepatocellular carcinoma and elevated baseline alpha-fetoprotein (REACH-2)	III	CPA, BCLC Stage C disease or BCLC Stage B disease not amenable to locoregional therapy or refractory to locoregional therapy. Prior sorafenib treatment		OS	399 pts	July 2015	April 2018	NCT02435433
VEGF	Sorafenib	TACE with or without Sorafenib	III	CPA or B7, first line treatment, branch not main PVI	Y	PFS	400 pts	October 2009	February 2018	NCT01004978
	Sorafenib	A randomized, controlled phase III trial of sorafenib with or without conventional TACE in patients with advanced HCC (STAH Study)	III	CPA or B7		OS	338 pts	February 2013	October 2017	NCT01829035
	Regorafenib	Study of regorafenib after sorafenib in patients with hepatocellular carcinoma (RESORCE)	III	CPA		OS	573 pts	May 2013	October 2016	NCT01774344
VEGF, FGF, PDGF, RET, KIT	Lenvatinib	A multicenter, open-label, phase 3 trial to compare the efficacy and safety of lenvatinib (E7080) <i>vs</i> sorafenib in first-line treatment of subjects with unresectable hepatocellular carcinoma	III	CPA, BCLC Stage B or C		OS	954 pts	March 2013	April 2016	NCT01761266
MET, RET, VEGF	Cabozantinib (XL 184)	Randomized controlled trial of XL184 <i>vs</i> placebo after sorafenib (CELESTIAL)	III	CPA		OS	760 pts	August 2013	October 2016	NCT01908426
MET	Tivantinib (ARQ197)	Study of tivantinib in subjects with inoperable hepatocellular carcinoma who have been treated with one prior therapy (METIV-HCC)	III	MET Diagnostic-High tissue		OS	368 pts	December 2012	June 2017	NCT01755767
	Tivantinib (ARQ197)	A randomized double-blind, placebo-controlled Japanese phase III trial of ARQ197 in hepatocellular carcinoma (HCC) (JET-HCC)	III	c-Met high in tumor sample, CPA		PFS	160 pts	January 2014	December 2016	NCT02029157
PD-1	Nivolumab	First line treatment with nivolumab <i>vs</i> sorafenib (CheckMate 459: CHECK-point pathway and nivolumab clinical trial evaluation 459)	III	CPA		TTP, OS	726 pts	November 2015	June 2019	NCT02576509
PD-1	Pembrolizumab	Study of pembrolizumab (MK-3475) <i>vs</i> best supportive care in participants with previously Systemically treated advanced hepatocellular carcinoma (MK-3475-240/KEYNOTE-240)	III	CPA, BCLC Stage C disease or BCLC Stage B disease not amenable to locoregional therapy		PFS, OS	408 pts	April 2016	April 2018	NCT02702401

PFS: Progression free survival; TTP: Time to progression; ORR: Overall response rate; OS: Overall survival; VEGF: Vascular endothelial growth factor; PDGF: Platelet derived growth factor; FGF: Fibroblast derived growth factor; PD-1: Programmed death-1; CP: Child-Pugh class; BCLC: Barcelona Clinic Liver Cancer; PVI: Portal vein invasion; TACE: Transarterial chemoembolization.

(AFP) levels and ramucirumab treatment response, found significantly improved median overall survival in patients who had elevated baseline AFP levels (≥ 400 ng/mL) ($P = 0.0059$)^[17]. Based on this preliminary result, the REACH-2 study has been designed to focus on patients with elevated baseline AFP (NCT02435433, Table 2).

Brivanib (BMS-582664) is a selective dual inhibitor of VEGF and FGF signaling pathways, which has inhibited angiogenesis and tumor growth in xenograft models of HCC^[18]. Brivanib has also shown clinical activity and good tolerability in patients with unresectable HCC. A multicenter, double-blind, randomized, placebo-controlled

Table 2 On-going National Cancer Institute-sponsored phase II trials

Target molecule	Molecule	Trial	Phase	Details	Primary outcome	Estimated enrollment	Start date	Estimated study completion date	Ref.
VEGFR	Ramucirumab	A study of LY2875358 in combination with ramucirumab (LY3009806) in participants with advanced cancer	I / II	Part A: Escalating doses of LY2875358 will be given in combination with a fixed dose of ramucirumab to evaluate the safety of the combination Part B: evaluation of safety and activity	Dose-limiting toxicities in part A ORR in part B	70 pts	March 2014	April 2017	NCT02082210
VEGF	Sorafenib	Sorafenib with Capecitabine and Oxaliplatin (SECOX)	II		PFS	52 pts	September 2007	December 2008 (status unknown)	NCT00752063
	Sorafenib	Sorafenib + mFOLFOX for hepatocellular carcinoma (HCC)	II	CPA, BCLC C or B not suitable for TACE	TTP	40 pts	January 2013	December 2017	NCT01775501
	Sorafenib	Sorafenib plus doxorubicin in patients with advanced HCC with disease progression on sorafenib	II	CPA	OS	30 pts	April 2013	April 2016	NCT01840592
	Sorafenib	Comparison study of sorafenib and 5-fluorouracil/mitomycin for metastatic HCC	II	Eligible patients have pulmonary metastasis and intrahepatic tumors controlled with locoregional therapies	PFS	40 pts	November 2010	July 2016	NCT01171482
	Sorafenib	Palliative treatment of HCC in patient with CHILD B cirrhosis (PRODIGE 21)	II	Sorafenib vs pravastatin vs sorafenib + pravastatin BCLC B or C	Time to radiologic progression	160 pts	November 2011	February 2016	NCT01357486
	Sorafenib	A study of LY2157299 in participants with advanced HCC	II	LY2157299 vs sorafenib vs placebo CPA	OS	120 pts	August 2014	December 2016	NCT02178358
	Axitinib	Axitinib as second-line treatment for advanced HCC	II	CPA	disease stabilization	45 pts	April 2011	December 2016	NCT01273662
TßRI	Galunisertib (LY2157299)	Galunisertib with nivolumab	I / II	A study of galunisertib (LY2157299) in combination with nivolumab in advanced refractory solid tumors and in recurrent or refractory NSCLC, HCC, or glioblastoma CPA	Phase 1b: MTD of Galunisertib in combination in combination with nivolumab	100 pts	October 2015	March 2019	NCT02423343
	Galunisertib (LY2157299)	A study of LY2157299 in participants with advanced HCC	II	A Randomized phase 2 study of LY2157299 vs LY2157299 - sorafenib combination vs sorafenib in patients with advanced HCC CPA	OS	120 pts	August 2014	October 2016	NCT02178358
	Galunisertib (LY2157299)	A study of LY2157299 in participants with HCC	II	The study consists of three parts: Part A: HCC participants with an increased alpha fetoprotein (AFP) level are treated with either 160 mg LY2157299 or 300 mg LY2157299; Part B: HCC participants with a normal AFP level are treated with 300 mg LY2157299; Part C: treatment-naïve HCC participants are treated with 160 mg LY2157299 + sorafenib or 300 mg LY2157299 + Sorafenib TEM 10 mg iv weekly + SOR 200 mg bid CPA, CPB ≤ 7	TTP Relation-ship of change in response biomarker to clinical benefit	190 pts	March 2011	October 2016	NCT01246986
mTOR	Temsirolimus plus sorafenib	Phase II combination of temsirolimus and sorafenib in advanced hepatocellular carcinoma	II		TTP	27 pts	September 2012	September 2017	NCT01687673

VEGF, PDGF, FGF	Nintedanib	Phase I / II comparison of efficacy and safety of BIBF 1120 and sorafenib in patients with advanced hepatocellular carcinoma	I / II	Nintedanib 200 mg bid or sorafenib 400 mg bid CPA	MTD in phase I TTP in phase II	125 pts	October 2009	January 2016	NCT01004003
PD-1	Pembrolizumab	Pembrolizumab (Keytruda) in advanced hepatocellular carcinoma	II	CP < 7, at sorafenib progression	Disease control rate	28 pts	March 2016	March 2019	NCT02658019
	Pembrolizumab	Study of Pembrolizumab (MK-3475) as monotherapy in adults with previously systemically treated advanced hepatocellular carcinoma (MK-3475-224/KEYNOTE-224)	II	CPA	ORR	100 pts	April 2016	November 2017	NCT02702414
	Pembrolizumab	Study of pembrolizumab (MK-3475) in participants with advanced solid tumors (MK-3475-158/KEYNOTE-158)	II	Multiple types of advanced (unresectable and/or metastatic) solid tumors that have progressed on standard of care therapy may be enrolled	ORR	1100 pts	December 2015	April 2018	NCT02628067
S100A9	Tasquinimod	A study with tasquinimod treating patients in four independent cohorts of hepatocellular, ovarian, renal cell and gastric cancers	II	BCLC C or B not amenable to locoregional therapy, CPA, previous treatment with sorafenib	PFS	201 pts	December 2012	February 2016	NCT01743469
PD-1	Nivolumab, nivolumab plus ipilimumab	Study to evaluate the effectiveness, safety and tolerability of nivolumab and the combination nivolumab plus ipilimumab in subjects with advanced liver cancer	I / II		Safety, ORR	600 pts	September 2012	July 2018	NCT01658878
PD-L1	MEDI4736	Biological/vaccine: MEDI4736 + tremelimumab Biological/vaccine: MEDI4736 Biological/vaccine: Tremelimumab	II		Safety	120 pts	October 2015	April 2018	NCT02519348

PFS: Progression free survival; TTP: Time to progression; ORR: Overall response rate; MTD: Maximum tolerated dose; OS: Overall survival; VEGF: Vascular endothelial growth factor; PDGF: Platelet derived growth factor; FGF: Fibroblast derived growth factor; PD-1: Programmed death-1; PD-L1: Programmed death ligand 1; CP: Child-Pugh class; BCLC: Barcelona Clinic Liver Cancer.

trial assessed brivanib in patients with HCC who had been treated with sorafenib^[19]. Median overall survival was 9.4 mo for brivanib and 8.2 mo for placebo (HR = 0.89, 95.8%CI: 0.69-1.15, $P = 0.3307$). Exploratory analyses showed a median TTP of 4.2 mo for brivanib and 2.7 mo for placebo (HR = 0.56, 95%CI: 0.42-0.76, $P < 0.001$), and an ORR by modified response evaluation criteria in solid tumors (RECIST) of 10% for brivanib and 2% for placebo (OR = 5.72). The most frequent treatment-related grade 3-to-4 adverse events for brivanib were hypertension (17%), fatigue (13%), hyponatremia (11%), and decreased appetite (10%). Brivanib was also compared to sorafenib in first-line treatment^[20]; median overall survival was 9.9 mo for sorafenib and 9.5 mo for brivanib; TTP, ORR, and Disease Control Rate also were similar between the study arms.

Lenvatinib (E7080) is an oral multi-targeted tyrosine kinase inhibitor of VEGFR1-3, FGFR1-4, PDGFR β , RET and KIT. A phase 1/2 open-label study evaluated the safety and efficacy of lenvatinib in 46 patients with advanced disease and Child Pugh A liver function status. Patients were treated with a starting dose of lenvatinib 2 mg daily (28-d cycles) until disease progression or development of unmanageable toxicities occurred. Median TTP was 12.8 mo (95%CI: 7.23-14.7), and median overall survival 18.7 mo (95%CI: 12.8-25.1). The most common adverse events were hypertension 76% (Gr 3, 54%), palmar-plantar erythrodysesthesia syndrome 61% (Gr 3, 7%), proteinuria 59% (Gr 3, 20%), anorexia 57% (Gr 3, 2%), thrombocytopenia 50% (Gr 3, 33%), and fatigue 48% (Gr 3, 0%). ORR was 37%, and 45.7% of patients had stable disease. Based on these phase 2 data, a global, randomized, open-label phase 3 trial is ongoing to determine if lenvatinib is non-inferior or superior compared with sorafenib in advanced HCC (NCT01761266; Table 1)^[21].

Axitinib, a potent, selective inhibitor of VEGF receptors, has been efficacious in phase 2 and 3 trials in previously treated patients with metastatic renal cell carcinoma. In preclinical studies, axitinib had antiangiogenic and anti-tumor activity in human tumor models. Phase II or phase III studies have found that axitinib has single-agent clinical activity in a range of tumor types, including renal cell carcinoma^[22], thyroid cancer^[23], non small-cell lung cancer^[24], and melanoma^[25]. Results of a phase II trial using 5 mg bid in second-line therapy of HCC have recently been published^[26]; median overall survival was not significantly improved in the axitinib/best

Supportive care (BSC) arm (12.7 mo) vs placebo/BSC (9.7 mo) (HR = 0.907, 95%CI: 0.646-1.274; one-sided stratified $P = 0.287$). Despite the absence of overall survival benefit, improvements in PFS, TTP, and clinical benefit rate with axitinib/BSC compared with placebo/BSC were shown. Most common adverse events with axitinib/BSC were diarrhea (54%), hypertension (54%), and decreased appetite (47%). Axitinib in second-line treatment is still being evaluated in a phase II ongoing trial (NCT 01273662; Table 2).

AGENTS TARGETING SIGNAL TRANSDUCTION

Agents developed to target signal transduction may act at the level of growth factor receptor or within the cell at the level of intracellular signaling. A number of strategies, including monoclonal antibodies and tyrosine kinase inhibitors, have been developed and tested in various phases of clinical trials.

A key signal transduction pathway implicated in HCC is the EGFR-RAS-MAPKK pathway. EGFR is frequently expressed in human HCC cell cultures and tumor tissues. The ligands EGF, hepatocyte growth factor (HGF), PDGF, and VEGF, among others, activate the RAS/MAPK signaling pathway and induce transcription of genes, such as *c-fos* and *c-jun*, which are key elements for cell proliferation^[27]. HCV core protein can directly activate the Raf/MEK/ERK cascade^[28].

Mutations of Raf and Ras are rare findings in HCC. Potent drugs blocking Ras/MAPK signaling are still at the exploratory phase, except for sorafenib, which can inhibit B-Raf at nanomolar concentrations.

Although phase II studies reported that erlotinib monotherapy had activity in patients with advanced HCC^[29,30], combining erlotinib with sorafenib did not enhance efficacy compared with sorafenib alone^[31]. Median overall survival was similar in the sorafenib plus erlotinib and sorafenib plus placebo groups (9.5 mo vs 8.5 mo, respectively, HR = 0.929, $P = 0.408$), as was median TTP (3.2 mo vs 4.0 mo, respectively; HR = 1.135, $P = 0.18$). In the sorafenib/erlotinib arm the ORR was higher (6.6% vs 3.9%, respectively, $P = 0.102$) than in the sorafenib/placebo arm, whereas the DCR was significantly lower (43.9% vs 52.5%, respectively, $P = 0.021$). Drug-related serious adverse events were similar in the two arms.

AZD6244 (selumetinib, ARRY-142886) targets the MAPK pathway by inhibiting MEK. AZD6244 is well tolerated but appears to have minimal activity in advanced HCC^[32].

Refametinib, an oral allosteric MEK inhibitor, has had anti-tumor activity in combination with sorafenib *in vitro* and *in vivo*. A phase II study evaluated efficacy and safety of refametinib plus sorafenib in Asian patients with HCC^[33] (NCT01204177). Anti-tumor activity was found in patients, however, dose

modifications were required due to adverse events, which occurred in almost all patients.

IGFR signaling has a major role in the regulation of fetal development, proliferation, differentiation, cell growth, and apoptosis. The IGF family consists of two ligands (IGF- I , IGF- II), two receptors, and six binding proteins. Ligand binding leads to the activation of the PI3K/Akt/mTor and MAPK pathways, among others. Dysregulation of IGFR signaling in HCC predominantly occurs at the level of IGF- II and the IGF-I receptor (IGF-1R)^[34,35]. Somatostatin reduces release of growth factors, such as IGF-1 or EGF^[36,37] and inhibits angiogenesis. The somatostatin analog octreotide, can be considered the first "biological" agent used in HCC. Several IGF-1R inhibitors are under investigation. The most advanced clinical antibody against IGF-1R is cixutumumab (IMC-A12), but cixutumumab monotherapy did not have clinically meaningful activity in an unselected HCC population^[38].

The RAS/MAPK pathway is activated in 50% of patients who have early stage HCCs and almost all of those with advanced-stage HCCs^[39,40]. Several compounds have been developed that target the c-MET/HGF signaling pathway, including antibodies against HGF or c-MET, or selective small-molecule inhibitors of c-MET^[41].

Cabozantinib (XL184) is a small-molecule inhibitor of the tyrosine kinases c-Met and VEGFR2, and has been shown to reduce tumor growth, metastasis, and angiogenesis. A phase III trial is underway in HCC patients who have received prior sorafenib^[42] (NCT01908426; Table 1).

Tivantinib (ARQ 197), a selective oral inhibitor of MET, has shown promising anti-tumor activity in HCC as monotherapy and in combination with sorafenib. Seventy-one patients were randomly assigned to receive tivantinib (38 at 360 mg twice-daily and 33 at 240 mg twice-daily); 36 patients were randomly assigned to receive placebo. TTP was longer for patients treated with tivantinib (1.6 mo, 95%CI: 1.4-2.8) than with placebo [1.4 mo (1.4-1.5); HR = 0.64, 90%CI: 0.43-0.94; $P = 0.04$]. For patients with MET-high tumors, median TTP was longer with tivantinib than with placebo [2.7 mo, 95%CI: 1.4-8.5 for 22 MET-high patients on tivantinib vs 1.4 mo (1.4-1.6) for 15 MET-high patients on placebo; HR = 0.43, 95%CI: 0.19-0.97, $P = 0.03$]. The most common grade 3 or worse adverse events in the tivantinib-treated group were neutropenia and anemia. Tivantinib at higher doses was associated with increased rate of grade 3 or worse neutropenia (21% vs 6%, respectively). Four patients treated with tivantinib died due to severe neutropenia^[43]. Results of two phase III trials of tivantinib in pre-treated MET-high HCC are awaited (Table 1).

LY2875358 is a novel humanized bivalent anti-MET antibody that has high neutralization and internalization activities, which can inhibit activation

of both HGF-dependent and HGF-independent MET pathways and tumor growth^[44]. A phase I / II trial with LY2875358 and ramucirumab is ongoing in patients with advanced cancer, including HCC patients (NCT02082210; Table 1).

AGENTS TARGETING THE PI3K/AKT/MTOR PATHWAY

The PI3K/Akt/mTOR pathway is a pivotal signaling cascade in cancer, particularly in HCC, and interferes with cell growth, proliferation, angiogenesis, and apoptosis^[45]. The pathway is activated through several receptor tyrosine kinases (RTKs) (e.g., EGFR or IGF1R). PI3K activity is additionally controlled by the tumor suppressor gene phosphatase and tensin homolog, which is mutated in a subgroup of HCCs. PI3K activates the serine/threonine kinase Akt, which phosphorylates and inactivates several pro-apoptotic proteins. The most relevant target downstream of Akt is mTOR, a central regulator of cell proliferation and angiogenesis^[45]. Phosphorylation of mTOR and its downstream targets were detected in human HCC. The PI3K/Akt/mTOR pathway is activated in 15%-41% of HCCs, and mTOR inhibitors had antineoplastic activity in experimental models of HCC^[46,47].

Several compounds which inhibit mTOR [sirolimus (rapamycin) and its analogues temsirolimus (CCI-779) and everolimus (RAD001)] are already used as immunosuppressants after liver transplantation, or for the treatment of renal cell carcinoma. Retrospective studies in patients who have had liver transplantation for HCC and concomitant immunosuppression with mTOR inhibitors have been reported^[48], with an outcome that suggests a prolonged overall survival and reduced tumor recurrence. Rapamycin is undergoing several trials intended to establish its role in this setting.

EVOLVE-1 was a randomized, double-blind, phase 3 study conducted with 546 adults with Barcelona Clinic Liver Cancer stage B or C HCC and Child-Pugh A liver function after treatment with sorafenib. Study subjects received everolimus, 7.5 mg/d, or matching placebo, both given together with best supportive care^[49]. No significant difference in overall survival was seen between treatment groups, with 303 deaths (83.7%) in the everolimus group and 151 deaths (82.1%) in the placebo group (HR = 1.05, 95%CI: 0.86-1.27, *P* = 0.68; median OS, 7.6 mo with everolimus, 7.3 mo with placebo). Median TTP with everolimus and placebo was 3.0 mo and 2.6 mo, respectively (HR = 0.93, 95%CI: 0.75-1.15), and disease control rate (DCR) was 56.1% and 45.1%, respectively (*P* = 0.01). The most common grade 3/4 adverse events for everolimus vs placebo were anemia, asthenia, and decreased appetite. No benefit was found for the combination of everolimus and pasireotide, a long-acting somatostatin multi-receptor ligand, in HCC^[50].

Also, no evidence was found that everolimus plus sorafenib is more efficacious than sorafenib alone^[51]. Median PFS (6.6 mo vs 5.7 mo), TTP (7.6 mo vs 6.3 mo), duration of disease stabilization (6.7 mo vs 6.7 mo), and overall survival (10 mo vs 12 mo) were similar in the sorafenib and sorafenib plus everolimus arms. Grade 3/4 adverse events were more common with the combination therapy. Everolimus has been tested also in association with TACE^[52].

There are no published data on phase II trials regarding temsirolimus alone in HCC; however, the combination of temsirolimus and bevacizumab was evaluated in 28 patients, with a favorable ORR of 19% and overall survival of 14 mo^[52]. A phase II study evaluating temsirolimus plus sorafenib is ongoing (NCT01687673; Table 2).

AGENTS TARGETING PROTEIN TURNOVER, CHROMATIN REMODELING, APOPTOSIS, AND CELL CYCLE CONTROL

The ubiquitin-proteasome pathway is the major nonlysosomal proteolytic system, and it triggers degradation of proteins involved in cell cycle progression, apoptosis, angiogenesis, and, particularly, NF- κ B activation. The 26S proteasome is a complex molecular machine that induces protein degradation and has become an attractive target for cancer therapy. Bortezomib (PS-341) reversibly and competitively inhibits the 26S proteasome, thus blocking multi-ubiquitinated protein degradation^[53]. Bortezomib was tested in a Phase I / II trial in 18 patients with advanced HCC and achieved stable disease in 46% of patients^[54]. In a phase II study enrolling 35 patients, no significant activity was shown and grade 3 and 4 adverse events were reported in 68% and 11% of treated patients^[55]. Moderate or severe liver dysfunction influenced the safety of bortezomib, with required dose adjustment to 0.7 mg/m²^[56]. Further development of the drug was probably restricted by inadequate consideration of this finding, which was particularly significant in HCC. Future research will focus on combination treatment strategies using bortezomib together with other targeted agents such as sorafenib^[57]. A phase II, open-label, multicenter study examined the efficacy of bortezomib (1.3 mg/m² IV on days 1, 4, 8, and 11) and doxorubicin (15 mg/m² IV on days 1 and 8) in 21-d cycles^[58]. The combination of the two drugs produced less grade 3 and 4 adverse events than that seen in the previous reported phase II study, but failed to demonstrate an ORR of at least 27% and had no encouraging efficacy results.

Evasion of apoptosis is one of the hallmarks of cancer. Several pro-apoptotic receptor agonists targeting the extrinsic apoptosis pathway [including the ligand recombinant human Apo2L/TNF-related apoptosis-inducing ligand (TRAIL)] are in development. Mapatumumab (HGS1012), a fully human agonist

monoclonal antibody targeting TRAIL receptor 1, in combination with sorafenib have been evaluated in a randomized, double-blind, placebo-controlled, phase II study^[59]. One hundred-one patients were randomized (placebo-sorafenib arm: $n = 51$; mapatumumab-sorafenib arm: $n = 50$). There was no significant difference in median TTP between the two arms [5.6 mo vs 4.1 mo, respectively; adjusted hazard ratio one-sided 90%CI: 1.192 (0, 1.737)]. No mapatumumab-related benefit was identified when TTP was evaluated in the stratified subgroups. The addition of mapatumumab to sorafenib did not result in improved secondary efficacy endpoints.

TGF- β SIGNALING

Galunisertib is a selective small-molecule inhibitor of T β RI. A Phase 1b/2 dose escalation and cohort expansion study will evaluate the safety and efficacy of galunisertib in combination with nivolumab in the treatment of advanced refractory solid tumors (Phase 1b) and in recurrent or refractory non-small cell lung cancer, HCC, or glioblastoma (Phase 2). This study is not yet open for participant recruitment (NCT02423343; Table 2). Galunisertib is being evaluated with or without sorafenib in an open label, 3-part, phase 2 study in patients with HCC. The study consists of 4 parts: Part A includes HCC patients with an elevated AFP level treated with galunisertib 160 mg/d (Arm A, $n = 37$) or 300 mg/d (Arm B, $n = 72$); Part B includes HCC patients with a normal AFP level treated with galunisertib 300 mg/d; Part C includes treatment-naïve HCC patients treated with galunisertib 160 or 300 mg/d plus sorafenib 800 mg/d; and Part D includes HCC patients (those intolerant to sorafenib, those whose disease progressed during treatment with sorafenib, or those naïve to treatment with sorafenib) treated with galunisertib 160 or 300 mg/d plus ramucirumab 8 mg/kg on days 1 and 15. Patients will be administered galunisertib daily for 14 d, followed by 14 d off (28-d cycle), with patients in Part C receiving sorafenib daily for 28 d. Adverse events and efficacy data have been presented for Part A^[60,61] (NCT01246986; Table 2): median TTP was 12 wk (90%CI: 6.6-12.6) in the overall population, with 12.1 wk in Arm A, 10 wk in Arm B, and 18.3 wk (90%CI: 6.6-42.4) in patients who were sorafenib naïve^[60,61]. A Phase 2 study evaluating galunisertib, sorafenib, or galunisertib with sorafenib in patients with advanced HCC is ongoing and recruiting patients (NCT02178358; Table 2).

IMMUNE SYSTEM MODULATORY DRUGS

The immune system plays an important role in the outcome and response to treatment of HCC patients: post-surgical tumor recurrence are reduced when dense lymphocytic tumor infiltration is present and T-cell responses against tumor antigens are associated

with patient survival^[62]. However, continued exposure to tumor antigens leads to T cell exhaustion, favored by intra-tumor expression of immune check-point inhibitors. In recent years we have witnessed the dawn of a new era in immunotherapy of HCC, with different approaches. While resistance inevitably develops to targeted agents, durable disease control is generally achieved by immunotherapies^[63]. Monoclonal antibodies that modulate the activity of immune check-point molecules, which are critical determinants of tumor evasion to immunity, have revolutionized the field of cancer immunotherapy and will probably do so with therapy of HCC also. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) plays a key role in downstaging the activity of T cells. Promising activity has been reported for tremelimumab, a CTLA-4 inhibitor: a phase II trial of tremelimumab in HCC patients has recently been reported (NCT01008358)^[64]. The study enrolled 21 chronic hepatitis C patients with Child-Pugh A or B cirrhosis and advanced HCC not amenable to percutaneous ablation or transarterial embolization. Partial responses were seen in 17.6% of the patients and 45% had stable disease for more than 6 mo.

Another immune checkpoint molecule, programmed death-1 (PD-1) inhibits effector T-cell responses within tissues. When programmed death-ligand 1 (PD-L1) binds to its receptor, PD-1, delivers a signal that inhibits TCR-mediated activation of IL-2 production and T cell proliferation. This is one of more potent mechanisms of escape of tumor cells to immune system. Clinical trials with two anti-PD-1 monoclonal antibodies, pembrolizumab (humanized IgG4) and nivolumab (fully human IgG4), are underway. A phase 1/2 study evaluating the effectiveness, safety and tolerability of nivolumab and the combination nivolumab plus ipilimumab is ongoing (NCT01658878, Table 2). That study plans to enroll three cohorts of patients stratified by viral etiology (HBV, HCV) and no viral infection. A phase III trial is comparing nivolumab to sorafenib in first-line treatment (NCT02576509; Table 1). Pembrolizumab is under investigation in several phase II studies (NCT02658019, NCT027024414, NCT02628067). A phase III trial (NCT02702401; Table 1) will give information on the efficacy of pembrolizumab in previously treated HCC patients. MEDI4736, another humanized IgG-1 κ monoclonal antibody which blocks PD-L1, is a subject of clinical trials. A Study of MEDI4736 with tremelimumab, MEDI4736 or tremelimumab monotherapy in unresectable HCC is recruiting participants (NCT02519348; Table 2).

Tasquinimod is a novel small-molecule inhibitor that targets the tumor microenvironment by controlling immunosuppressive, pro-angiogenic and pro-metastatic functions of regulatory myeloid cells (also called myeloid-derived suppressor cells)^[65]. It binds to and inhibits the interactions of S100A9, an immunomodulatory protein that promotes tumor development. Tasquinimod inhibits the growth and metastasis of tumor cells *in vitro* and

in vivo^[65]. A phase II study is ongoing in treatment of several types of tumors, including HCC (NCT01743469; Table 2).

DISCUSSION

Upon review of medical research in HCC, we find some new molecules disappearing after phase I / II studies without published results; most drugs in development, with poor results; and only a few new drugs surviving at selection with positive outcomes. HCC is a difficult disease to study because of its clinical and molecular heterogeneity and the presence of underlying liver cirrhosis. Studies conducted during the past decade have defined the main genomic subclasses of HCC: a primary classification of tumors consists of proliferative and non-proliferative genotypes, each comprising approximately 50% of patients^[5]. Overall, the proliferative subclass is enriched by activation of classic RAS, mTOR and/or IGF signaling and is associated with a poorer outcome than that of non-proliferative phenotypes. From an epidemiological standpoint, HBV-related HCCs usually cluster within the proliferative subclass, whereas alcohol-related and HCV-related HCCs are enriched in the non-proliferative subclass^[5]. Probably each tumor subclass is linked to a specific mutation signature profile and may benefit by an approach different from that for the other subclass. Therefore, it is crucial to select drugs that interfere with oncogenic drivers and not bystander mutations. Similarly to what happened in other tumors, dependency of tumor cells on activated oncogenes or loss of tumor suppressors has been the key to identifying drugs capable of producing favorable clinical results. Thus far, no main driver and pathway has been identified in HCC. However, several studies have provided a broad picture of the mutational profile in HCC and identified an average of 30-40 mutations per tumor, among which 5-8 might be driver mutations^[5]. There is a rationale for blocking complementary pathways activated in HCC^[66]. Along with the identification of these pathways is the need for tumor tissue to assess markers predictive for response. As in other types of tumors, the identification of biomarkers could predict response to a date therapy. Perhaps a more aggressive tumor phenotype could particularly benefit, if discovered early, from local interventions followed by maintenance medical treatment and, in later stages, by a chemo-targeted approach, either sequential or combined.

Sorafenib has changed the medical approach to advanced HCC; however, data supporting its use are not based on response rate (2% partial response) and improvements in quality of life and cancer symptoms, but only on a modest survival advantage^[6]. It is also important to appreciate that there is a difference between criteria of clinical studies and general practice: the majority of trials select Child A

and ECOG 0-1 patients, which do not represent the real population of HCC patients. Most studies also lack a stratification taking into account factors like portal invasion and metastases^[5]. Specific phase II studies exploring potential liver-related toxicities of new agents are required in patients with cirrhosis and HCC before testing in phase III randomized controlled trials. We frequently found that increased aspartate aminotransferase concentration, thrombocytopenia, hyperbilirubinemia, and ascites are cited among adverse events, but whether these are due to drug toxicity only or to progression of liver disease is not easy to determine. In clinical practice, usually these events are unchanged or worsen after stopping a drug because they are simply related to the evolution of tumor/cirrhosis. Better supportive liver care in chronic hepatitis/cirrhosis can help tumor treatment; however, thus far, only control of viral infection, through the use of new antiviral agents, might significantly impact on the outcomes of HCC treatment. A recent systematic review concluded that there are few data on the supportive-care needs of patients with advanced liver disease and cirrhosis^[67]. Activity of biological therapies at doses different from those registered is another field of investigation^[11].

Thus far, efforts at treating HCC have been concentrated on advanced HCC because transplantation, surgery, and local treatments gave the best chance of cure in early HCC. However, attempts to reduce recurrences are ongoing, especially with sorafenib in association with local therapies.

According to American Association for the Study of Liver Diseases and Journal of the National Cancer Institute guidelines^[68], new molecules tested in the first-line setting need to be combined with the standard of care, sorafenib, to demonstrate superiority^[5]. However, only one randomized controlled trial, which tested sorafenib plus erlotinib vs sorafenib alone, was planned according to this recommendation. Furthermore, response criteria must be chosen carefully. Tumor shrinkage is not a valid end point for HCC, especially since tumor activity of targeted therapies is cytostatic rather than cytotoxic^[69]. Overall survival is considered as the only valid primary end point, even in the phase II setting^[5].

CONCLUSION

Thus far, no novel, fully effective drug in the treatment of HCC has been produced. HCC remains a complex disease. The lack of a driver oncogene and the presence of underlying liver cirrhosis are factors which are most responsible for the frequently unsuccessful results with novel drugs. Insights into signaling pathways could help in identifying drugs likely to be effective. We feel that a unique targeted therapy for HCC probably does not exist and a tailored medical approach is the best that can be offered at the moment.

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2016 Hepatocellular Carcinoma: Global View

Alpha-fetoprotein-targeted reporter gene expression imaging in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers in Eastern Asia, and its incidence is increasing globally. Numerous experimental models have been developed to better our understanding of the pathogenic mechanism of HCC and to evaluate novel therapeutic approaches. Molecular imaging is a convenient and up-to-date biomedical tool that enables the visualization, characterization and quantification of biologic processes in a living subject. Molecular imaging based on reporter gene expression, in particular, can elucidate tumor-specific events or processes by acquiring images of a reporter gene's expression driven by tumor-specific enhancers/promoters. In this review, we discuss the advantages and disadvantages of various experimental HCC mouse models and we present *in vivo* images of tumor-specific reporter gene expression driven by an alpha-fetoprotein (AFP) enhancer/promoter system in a mouse model of HCC. The current mouse models of HCC development are established by xenograft, carcinogen induction and genetic engineering, representing the spectrum of tumor-inducing factors and tumor locations. The imaging analysis approach of reporter genes driven by AFP enhancer/promoter is presented for these different HCC mouse models. Such molecular imaging can provide longitudinal information about carcinogenesis and tumor progression. We expect that clinical application of AFP-targeted reporter gene expression imaging systems will be useful for the detection of AFP-expressing HCC tumors and screening of increased/decreased AFP levels due to disease or drug treatment.

Key words: Alpha-fetoprotein; Hepatocellular carcinoma; Molecular imaging; Reporter gene; Tumor-specific enhancer/promoter

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Core tip: It is essential to establish an appropriate animal model of hepatocellular carcinoma (HCC) for monitoring the disease progression and evaluating therapeutic interventions with anticancer drugs. Reporter gene-based molecular imaging can elucidate tumor-specific events or processes through acquisition of images of reporter gene expression driven by tumor-specific enhancers/promoters. In this paper, we describe the advantages and disadvantages of various animal models of HCC and present images of *in vivo* reporter gene expression controlled by alpha-fetoprotein enhancer/promoter in the various HCC animal models.

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INTRODUCTION

Molecular imaging methods can provide novel insights into biological and physiological processes through visualization of cellular or molecular events in a living organism. The greatest significance of molecular imaging methods lies in their ability to non-invasively and repetitively obtain longitudinal and quantitative biological information in an *in vivo* setting^[1]. Moreover, when compared to analytical methods involving biopsied tissues, the non-invasive nature of molecular imaging is less time-consuming and provides more reliable inferences to the organism.

When applied to evaluate the efficacy and therapeutic mechanism of new drugs, molecular imaging can help to determine biochemical or metabolic changes *in vivo* by use of Tc-99m labeled annexin V (for apoptosis), Tc-99m labeled vascular endothelial growth factor or Cu-64/In-111/Tc-99m labeled arginylglycylaspartic acid peptide (for angiogenesis), Cu-64 labeled methylthiosemicarbazone or F-18 labeled fluoroazomycin arabinoside (for hypoxia), or F-18 labeled 2-deoxy-D-glucose, F-18 labeled 3'-deoxy-3'-fluorothymidine or F-18 labeled fluoroethyltyrosine (for glucose, nucleotide or amino acid metabolism)^[2]. The pharmacokinetic and pharmacodynamic properties of new drugs, including tissue biodistribution at designated time points and the binding affinity of ligands to their target receptors, can also be determined by nuclear

medicine imaging using radioisotope-labeled drugs^[3]. At present, the applicability of molecular imaging methods to drug development is increasing in scale and scope, with the added benefit of providing further clarification of basic biological phenomena^[4].

The currently available molecular imaging methods are classified into the following categories according to their technical modalities: optical imaging (fluorescence and bioluminescence); magnetic resonance imaging (MRI); nuclear medicine imaging, including scintigraphy, positron emission tomography (PET) and single-photon emission computed tomography (commonly known as SPECT); and others, including ultrasound imaging and photoacoustic imaging. Optical imaging has high sensitivity, but its clinical utility is limited due to its low depth of penetration. MRI has high resolution and excellent soft-tissue contrast, but poor sensitivity and the expensive cost of the MRI equipment have proven prohibitive to its widespread application. Nuclear medicine imaging has high sensitivity and unlimited depth penetration, but again cost of the equipment is prohibitive and its limited spatial resolution is another limiting factor. Ultrasound imaging has high spatial/temporal resolution and a much more affordable (relatively low cost) profile, but is limited to vascular compartments and its reliability can be operator-dependent^[5,6]. Therefore, several multimodality imaging instruments, such as PET/CT, PET/MR, and optical imaging/computed tomography (CT), have been developed to overcome the distinct disadvantages of each and now play an important role in basic and clinical research.

Molecular imaging with reporter gene expression (also known as molecular genetic imaging) is defined as an imaging method that makes use of reporter gene expression in a target cell or tissue. Various imaging reporter genes have been developed for optical and nuclear medicine imaging, with the most popular being those encoding firefly luciferase, a variety of fluorescent proteins, the herpes simplex virus type 1 thymidine kinase (HSV1-tk) and the sodium iodide symporter (NIS)^[5,7]. To localize and track target cells *in vivo*, a reporter gene driven by a strong constitutive promoter, such as that of cytomegalovirus (commonly referred to as CMV), is first introduced into target cells. The reporter gene-expressing target cell is injected into a living organism and images are then acquired at designated time points following target cell injection. Because the *HSV1-tk* gene is applied for therapy as well as imaging, this gene expression can be monitored by visualization with an imaging technique^[5]. Therefore, combining molecular imaging and gene therapy can allow for real-time evaluation of location and duration of expression of a therapeutic gene, and successful application of this method in clinical practice has been reported^[8,9].

Because tumor-specific enhancers/promoters, such as the alpha-fetoprotein (AFP) enhancer/promoter

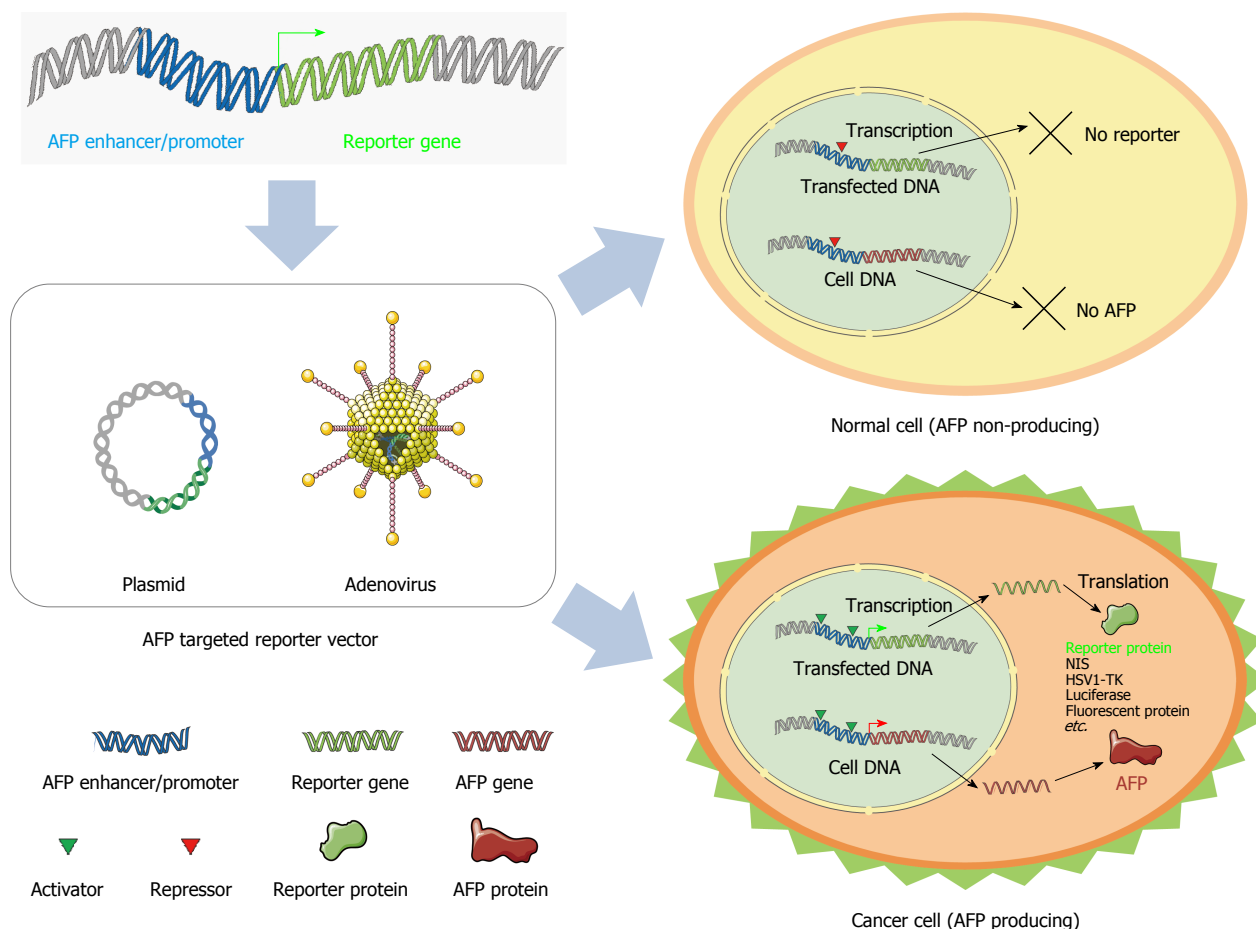


Figure 1 Cancer-specific reporter gene expression mediated by the alpha-fetoprotein enhancer/promoter. A reporter gene was delivered into cells using a vector system, such as a plasmid or adenovirus. In alpha-fetoprotein (AFP)-producing cancer cells, the level of the transcriptional activator of the *AFP* gene is high and thus the AFP enhancer/promoter is active. In contrast, in normal (non-cancer, non-AFP producing) cells, expression of the transcriptional repressor of the *AFP* gene is high and the *AFP* enhancer/promoter is inactive. The reporter protein is activated only in cancer cells with a high AFP level. AFP: Alpha-fetoprotein; HSV1-TK: Herpes simplex virus type 1 thymidine kinase; NIS: Sodium iodide symporter.

(Figure 1) and human telomerase reverse transcriptase, are highly active in cancer cells but not in non-cancer cells, they may serve as markers of cancer pathogenesis and progression that can be monitored by imaging of reporter gene expression. Indeed, several studies have already shown the utility of such reporter gene expression imaging using various tumor-specific enhancers/promoters, including survivin, mucin-1, carcinoembryonic antigen, prostate specific antigen and progression elevated gene-3; these studies are summarized in Table 1^[10-20].

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and is currently ranked fifth in incidence and second in mortality among males^[21]. Fetal liver cells normally express AFP, but expression decreases rapidly after birth. In adults, however, AFP expression has been found to resume under abnormal conditions, including liver cancer and cirrhosis. Although the diagnostic accuracy of AFP in small HCC (< 3 cm) is limited, it has been a useful and convenient diagnostic tool for HCC since the 1970s^[22]. Thus, many of the studies of HCC-specific imaging have used AFP-targeted reporter gene expression.

It is essential to establish an HCC animal model for monitoring of HCC progression and therapeutic interventions. In this review, we discuss methods for preparation of experimental HCC mouse models and the particular advantages and disadvantages of each. The AFP-specific reporter gene system, *in vivo* imaging applications for hepatocarcinogenesis and detection of AFP-positive HCC are also presented in the context of the mouse models.

EXPERIMENTAL HCC MOUSE MODELS

Mouse models of HCC development are established by xenografting, carcinogen induction or genetic engineering, according to the tumor-inducing factor and tumor location of research interest. Table 2 provides an overview of the various HCC mouse modeling methods and their key advantages and disadvantages.

Xenograft HCC models

Xenograft HCC models involve inoculation of cultured HCC cells into immune-deficient mice, such as the

Table 1 Reporter gene expression imaging with tumor-specific enhancers/promoters

Enhancer/promoter	Reporter gene (delivery method)	Imaging modality	Targeted tumor	Ref.
Survivin	<i>hNIS</i> (adenovirus)	Gamma camera	Ectopic xenograft PC-3 (prostate cancer) HepG2 (hepatoma) A375 (melanoma)	Huang <i>et al</i> ^[10]
	<i>fluc</i> (adenovirus)	Bioluminescent imaging	Orthotopic xenograft McA-RH7777 (rat hepatoma)	Ahn <i>et al</i> ^[11]
Mucin-1	<i>fluc</i> (adenovirus)	Bioluminescent imaging	Ectopic xenograft (metastasis) KPL-1 (breast cancer)	Huyn <i>et al</i> ^[12]
Hepatocarcinoma-intestine-pancreas (HIP)	<i>NIS</i> (adenovirus)	SPECT-CT	DEN-induced HCC (rat)	Hervé <i>et al</i> ^[13]
Prostate specific antigen (PSA)	<i>fluc/HSV1-sr39tk</i> (adenovirus)	Bioluminescent imaging/ PET	Ectopic xenograft LNCaP (prostate cancer)	Iyer <i>et al</i> ^[14] Jiang <i>et al</i> ^[15]
Carcinoembryonic antigen (CEA)	<i>HSV1-tk</i> (adenovirus)	Gamma camera	Ectopic xenograft MOD (murine breast cancer)	Qiao <i>et al</i> ^[16]
	<i>hNIS</i> (adenovirus)	Gamma camera	Ectopic xenograft TT (medullary thyroid cancer)	Spitzweg <i>et al</i> ^[17]
Progression elevated gene (PEG)-3	<i>fluc/HSV1-tk</i> (plasmid)	Bioluminescent imaging/ SPECT-CT	Ectopic xenograft (metastasis) MeWo (melanoma) MDA-MB-231 (breast cancer)	Bhang <i>et al</i> ^[18]
Telomerase reverse transcriptase (TERT)	<i>hNIS</i> (plasmid)	SPECT-CT	Ectopic xenograft Hep3B (hepatoma)	Kim <i>et al</i> ^[19]
	<i>GFP</i> (lentivirus)	Fluorescence imaging	Ectopic xenograft HepG2 (hepatoma) SGC-7901 (gastric cancer) SW480 (colon cancer)	Yu <i>et al</i> ^[20]

fluc: Firefly luciferase; GFP: Green fluorescent protein; hNIS: Human sodium iodide symporter; HSV1-TK: Herpes simplex virus type 1-thymidine kinase; PET: Positron emission tomography; SPECT-CT: Single-photon emission computed tomography-computed tomography.

Table 2 Experimental hepatocellular carcinoma animal models

Category		Inducing factor	Latency	Advantages	Disadvantages
Xenograft	Ectopic Orthotopic	Cancer cell line	Several weeks	Fast and easy modeling Easy detection of tumorigenesis	Less clinical relevancy Only reflect the characteristics of the selected cells
Carcinogen-induced		DEN	5-10 mo	More clinical relevancy	Lengthy time and high cost to model
Genetically engineered		HBV-derived	12-24 mo	Uncovering the molecular mechanisms of hepatocarcinogenesis	Difficult to detect tumorigenesis
		HCV-derived	12-24 mo	Closely mimic the pathophysiological features of human HCC	
		Oncogene-derived	Several weeks		

DEN: Diethylnitrosamine; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

athymic or severe combined immunodeficiency (SCID) strains. Xenograft models can be generated ectopically, by subcutaneous injection of HCC cells into extrahepatic locations such as the flank or thigh, or orthotopically, by direct injection into the liver; the latter approach more accurately reflects the *in vivo* tumor environment^[23]. Xenograft models are considered an easy, rapid and efficient means by which to demonstrate proofs of concept when appropriate cell lines are selected^[24]. However, this model has less clinical relevancy than autochthonous HCC models (which are beyond the scope of this review and not discussed herein).

Carcinogen-induced HCC models

Carcinogens are subdivided into two classes, namely

the genotoxic and non-genotoxic (or epigenetic) types. Genotoxic carcinogens irreversibly damage DNA, leading to genetic alterations and interference with normal biological processes; these types of carcinogens comprise chemical or non-chemical agents, such as radiation (ultraviolet or ionizing) and viruses. Non-genotoxic carcinogens do not directly adduct DNA structure, but alter cellular metabolism and promote uncontrolled malignant division. In general, the effect of carcinogens is insidious because they may not be immediately toxic^[23,25-27]. Carcinogen-induced HCC mouse models are principally used for evaluation of human hepatocarcinogenesis, and these studies must take into consideration that HCC in both human and mouse can be affected by additional contributing

factors - *e.g.*, sex, age and genetic background^[28].

Several chemical carcinogens can induce HCC formation to establish the mouse model system; the most commonly used are diethylnitrosamine (DEN)^[29], aflatoxin^[30], thioacetamide^[31], and carbon tetrachloride^[32]. Among these, DEN is the preferred agent, since the other chemical carcinogens have human toxicity and yield low tumor incidence and delayed carcinogenesis^[23]. The carcinogenic property of DEN arises from its alkylation of cellular DNA^[33] and generation of reactive oxygen species^[34]. DEN is generally administered to the mice as a single dose between postnatal days 12-15. The single-dose DEN administration to generate HCC must consider the dosage applied as well as the sex and age of the animal^[35-37]. Research groups using the single-dose protocol that was originally described by Vesselinovitch and Mihailovich have reported that intraperitoneal injection of DEN results in generation of HCC in 100% of B6C3F1 male mice after 44 wk^[38-40]. In addition, the role of androgen receptor (AR) status has been demonstrated as important in DEN-induced murine hepatocarcinogenesis^[41].

Genetically engineered HCC models

As a consequence of the known significant differences between mouse and human carcinogenesis, carcinogen-induced HCC mouse models are not appropriate for determining the molecular mechanisms of human hepatocarcinogenesis. In contrast, genetically engineered mouse (GEM) models closely mimic the pathophysiological and molecular features of human HCC^[42]. GEM modeling, then, is more feasible for studies to understand the complexities of human diseases, such as HCC, and for assessment of the molecular mechanisms of tumor generation, progression and maintenance in particular^[43]. GEM models have already been successfully used in investigations of specific genes and their interactions with other genes^[28]. Numerous GEMs have been developed to study liver tumorigenesis in mice, with the most frequently used ones involving overexpression (transgenic mice) or deletion (knockout mice) of a specific gene^[44]. Moreover, these GEM models have been developed by various techniques, including pronuclear injection (additive transgenesis), homologous recombination (targeted transgenesis using embryonic stem cell technology), and RNA interference (to generate knockdown mice)^[45].

More than 80% of HCCs in humans are attributable to infection with the hepatitis B virus (HBV) and/or the hepatitis C virus^[46]. The genome of HBV is characterized by four overlapping open reading frames, which encode surface, core, polymerase and X (HBx) proteins. HBx transgenic mice are more sensitive to a single DEN-injection than their non-transgenic counterparts^[47,48]. In general, mutation or overexpression of the *Myc* gene is associated with

tumorigenesis, including that of HCC. A mouse model with overexpression of the human transforming growth factor- α (TGF- α) under control of the methallothionein (MT) 1 promoter develops HCC. Concordantly, it has been reported that *Myc*- and TGF- α over-expressing transgenic mice are genetically close to human HCC and that the overexpression profile is related with prognosis^[49]. In transgenic mice with over-expression of epidermal growth factor (EGF) under the control of the albumin promoter, the overexpression of secreted EGF leads to generation of multiple highly malignant hepatic tumors^[50]. In addition, other DNA viruses, such as simian vacuolating virus 40 (SV40), have the potential to cause tumors^[51].

AFP-TARGETED REPORTER GENE EXPRESSION IMAGING IN HCC MOUSE MODELS

AFP is a biomarker of HCC, and much progress has been made in our understanding of the mechanistic underpinnings of AFP expression in HCC. Because the *AFP* gene becomes re-expressed in HCC, tumorigenesis can be monitored using reporter gene expression imaging. However, few AFP-targeted reporter gene-imaging studies have been published; those studies on reporter gene imaging driven by AFP enhancer/promoter in HCC mouse models are presented in Table 3^[52-60].

Xenograft HCC models

Xenograft tumor models are preferred for use in cancer research because of their ease of tumor establishment. Several studies of AFP-targeted imaging or therapeutic effects in xenograft ectopic HCC mouse models established by the ectopic approach have been reported which carried out assessment via optical or nuclear medicine imaging modalities. A reporter gene under the control of the AFP enhancer/promoter can be delivered into cells using a plasmid vector (cell transfection) or adenoviral vector (systemic or intratumoral administration) system.

Jin *et al.*^[52] and Willhauck *et al.*^[53] used a plasmid system under the control of the AFP enhancer/promoter to achieve stable *NIS* gene expression in an HCC cell line. The AFP-targeted *NIS* gene expression system functioned well *in vitro* and *in vivo*, indicating the feasibility of HCC-specific reporter gene expression systems for diagnosis and therapy of AFP-positive HCC. However, this plasmid system proved to be limited in terms of its ability to deliver the reporter gene to the target region *in vivo* due to use of a plasmid-transfected HCC cell line. In other studies, an adenoviral vector system using the AFP enhancer/promoter was injected intratumorally in a xenograft HCC model established by the ectopic approach^[54,55]. Those results indicated the potential of *in vivo* AFP-

Table 3 Reporter gene expression imaging by alpha-fetoprotein enhancer/promoter system

Tumor model	Mouse strain	Induction of HCC	Reporter gene	Delivery system	Injection route	Ref.
Xenograft	NOD/SCID	HuH-7 cells	<i>hNIS</i>	Adenovirus ¹	IV	Kim <i>et al</i> ^[56]
	BALB/c nude		<i>TK/fLuc</i>	Adenovirus ¹	IT	Park <i>et al</i> ^[57]
	NOD/SCID		<i>fLuc</i>	Adenovirus ¹	IV	Kim <i>et al</i> ^[58]
	BALB/c nude	HepG2 cells	<i>hNIS</i>	Plasmid ²	-	Jin <i>et al</i> ^[52]
	CD-1 nude		<i>hNIS</i>	Adenovirus ¹	IT	Klutze <i>et al</i> ^[54]
	BALB/c nude		<i>hNIS</i>	Adenovirus ¹	IT	Ma <i>et al</i> ^[55]
Carcinogen-induced	NMRI nude		<i>hNIS</i>	Plasmid ²	-	Willhauck <i>et al</i> ^[53]
	C57BL/6	DEN	<i>fLuc</i>	Adenovirus ¹	IV	Kim <i>et al</i> ^[58]
	FVB/N	DEN	<i>TK/fLuc</i>	Transgenesis ³	-	Lu <i>et al</i> ^[59]
	C57BL/6	DEN	<i>fLuc</i>	Transgenesis ³	-	Park <i>et al</i> ^[60]

¹Adenovirus: *in vivo* delivery of reporter gene; ²Plasmid: use of reporter gene transfected cells; ³Transgenesis: use of transgenic reporter mice. DEN: Diethylnitrosamine; fLuc: Firefly luciferase; hNIS: Human sodium iodide symporter; IT: Intratumoral injection; IV: Intravenous injection; NOD/SCID: Non-obese diabetic/severe combined immunodeficiency; TK: Thymidine kinase; HCC: Hepatocellular carcinoma.

targeted imaging and a radiotherapeutic approach in HCC.

Kim *et al*^[56] and Park *et al*^[57] reported *in vivo* systemic delivery of an adenoviral vector system with a reporter gene (NIS, fLuc or HSV1-tk) driven by the AFP enhancer/promoter. Those studies showed that targeted imaging and therapy through systemic delivery of adenoviral vector was possible. Also, Kim *et al*^[58] introduced AFP-targeted bioluminescent imaging performed using adenovirus in xenograft and carcinogen-induced HCC tumor models. Thus, the adenovirus system is capable of facilitating AFP-targeted imaging and therapy in carcinogen-induced HCC as well as in xenograft tumor models.

Carcinogen-induced HCC models

Generally, carcinogen-induced HCC animal models can be established upon exposure of genetically susceptible mice to a variety of chemical carcinogens, such as DEN. Induction of HCC using carcinogens is more difficult than by the xenograft approach because of the longer generation time and greater difficulty of verification of AFP expression.

Lu *et al*^[59] reported that a hepatocarcinogenesis reporter (HCR) transgenic mouse model enables monitoring of tumorigenesis by bioluminescent and nuclear medicine imaging. In their HCR mouse model, the HSV1-tk and fLuc genes were concurrently expressed under the control of the AFP enhancer/promoter. The bioluminescent signal was then detected during the early stage of DEN-induced HCC, prior to neoplastic transformation. Detection at later stages revealed high expression of fLuc and HSV1-tk. In another study, Park *et al*^[60] demonstrated non-invasive monitoring of AFP expression and DEN-induced hepatocarcinogenesis using bioluminescent imaging in transgenic mice with the fLuc gene under the control of the AFP enhancer/promoter. These two transgenic mouse models enabled *in vivo* monitoring of AFP expression throughout the entire disease course and lifetime of the afflicted animal. These studies suggested the usefulness of AFP-targeted reporter

gene expression imaging for non-invasive *in vivo* evaluation of hepatocarcinogenesis.

CONCLUSION

We have presented here the various experimental HCC animal models, including xenograft, carcinogen-induced and genetically engineered HCC models, and discussed their characteristics. Non-invasive real-time *in vivo* molecular imaging of reporter gene expression under the control of tumor-specific enhancers/promoters can provide longitudinal information about carcinogenesis and tumor progression. We expect that AFP-targeted reporter gene expression imaging systems will be applied for the detection of AFP-expressing HCC tumors and screening of increased/decreased AFP levels due to disease or drug treatment.

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2016 Liver Transplantation: Global view

Role of NK, NKT cells and macrophages in liver transplantation

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Abstract

Liver transplantation has become the treatment of

choice for acute or chronic liver disease. Because the liver acts as an innate immunity-dominant organ, there are immunological differences between the liver and other organs. The specific features of hepatic natural killer (NK), NKT and Kupffer cells and their role in the mechanism of liver transplant rejection, tolerance and hepatic ischemia-reperfusion injury are discussed in this review.

Key words: Liver transplantation; Natural killer cells; Kupffer cells; Graft rejection; Ischemia-reperfusion injury

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Core tip: Liver transplantation has become the treatment of choice for acute or chronic liver disease. There are immunological differences between the liver and other organs. The specific features of selected hepatic immune cells, such as natural killer (NK), NKT and Kupffer cells, and their role in the mechanism of liver transplant rejection, tolerance and hepatic ischemia-reperfusion injury are discussed in this review.

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INTRODUCTION

Previous studies have intensively investigated immunological processes after liver transplantation. Ischemia-reperfusion injury and graft rejection are two major causes for poor outcomes following liver transplantation. Both processes are triggered and maintained by

immune cells. The specific features of hepatic natural killer (NK), NKT and Kupffer cells and their role in the mechanism of liver transplant rejection and hepatic ischemia-reperfusion injury based on the current literature are discussed in this review.

LIVER TRANSPLANTATION AND IMMUNOLOGICAL PROCESSES

During the last 50 years liver transplantation has become the treatment of choice for acute or chronic liver disease^[1]. The main indications for liver transplantations are primary liver tumors, chronic viral hepatitis, alcohol-related cirrhosis, chronic cholestatic liver disease, autoimmune hepatitis, vascular and metabolic disorders^[2,3]. With an overall 5-year survival of approximately 70%, the life expectancy of liver transplant recipients is lower than the general population^[4,5]. In addition to de novo malignancies, infections, cardiovascular or renal disease, ischemia-reperfusion-injury of the liver graft and graft rejection are important immunological processes responsible for long-term graft and patient survival after liver transplantation.

The liver acts as an innate immunity-dominant organ, therefore, hepatic immune cells provide the first line of defense against pathogens, infections or tumors^[6]. In addition to NK cells, macrophages (Kupffer cells), NKT cells and $\gamma\delta$ T cells, there are a large number of innate immune cells within the liver^[7,8]. In humans, NK cells are the most abundant lymphocyte population in the liver^[9].

Two specific and immunologically important processes occur after liver transplantation: (1) donor liver-resident cells enter the blood flow of the recipient; and (2) recipient immune cells invade the donor graft. This phenomenon occurs early after transplantation^[10-12]. It has been shown, that after liver transplantation, donor specific liver NK cells are detectable in the recipients' circulation up to two weeks after liver transplantation^[1,12]. The liver has been described as an immunotolerant organ^[6,13]. This immunotolerance is believed to be responsible for the lower levels of immunosuppressive drugs needed and the lower rate of allograft rejection after liver transplantation compared to other solid organ transplantations^[1,14]. This is reflected by the withdrawal of immunosuppression, in some cases, after liver transplantation, and the aim to wean patients from immunosuppressive drugs as soon as possible^[1,15]. In addition, it has been shown that hepatic grafts might facilitate the acceptance or reverse the rejection of other transplanted grafts, *e.g.*, heart or kidney after liver transplantation^[16].

MECHANISM OF GRAFT REJECTION

Acute graft rejection is a combined response of the

adaptive (cellular immunity) and humoral immune system (secreted antibodies by activated B cells) in combination with the innate immune system (phagocytosis). Furthermore, early organ rejection can be distinguished from late organ rejection. Wiesner *et al*^[17] suggested the following risk factors: lower recipient age, cold ischemia duration longer than 15 h, donor age and fewer human leukocyte antigen (HLA)-DR matches. T cells were believed to be solely responsible for graft rejection. However, there is increasing evidence that other cells of the adaptive immune system, such as NK cells, are also responsible and interact with T cells during graft rejection^[1,18,19]. In contrast to other solid organ transplantations, HLA cross-matching is not routinely performed prior to liver transplantation despite recent studies suggesting HLA markers, such as killer cell immunoglobulin-like receptors, influence the outcome of liver grafts^[20-22]. To date, clinical experience, analysis of immunosuppressive drug levels, serum liver enzymes and histological assessment have been used as markers to diagnose graft rejection^[23]. During acute graft rejection, mononuclear cells infiltrate the portal tract and the accumulation of activated lymphocytes leads to the secretion of chemokines and cytokines and subsequently, liver tissue injury^[24]. Furthermore, bile duct injuries and venous endophlebitis are histological features for the diagnosis of graft rejection^[25]. Although the exact chemotactic triggers are still under investigation, it is postulated that for NK cells, CCL3 leads to NK cell migration to the site of liver injury^[26-28].

MECHANISMS OF HEPATIC ISCHEMIA-REPERFUSION INJURY

During organ donation and transplantation, the liver undergoes trauma due to cold and non-perfused storage, warm ischemia and finally, engraftment. During ischemia-reperfusion liver injury, one important issue is organ preservation, which is initially triggered by endothelial cell injury and causes an acute inflammatory response that involves Kupffer cells, hepatocytes and hepatic stellate cells^[29]. Furthermore, cell death is caused by oxidative stress, which leads to increased microcirculatory disturbances, cell dysfunction and inflammation^[30,31]. To avoid organ damage due to organ preservation, several modifications have been investigated, such as perfusion solutions^[32-34], use of antioxidants^[35], vasodilators^[36,37], hydrogen gas^[38], or *ex-vivo* liver perfusion systems^[39-41]. Ischemia-reperfusion injury is crucial for initial and long-term organ function^[42]. Hepatic ischemia-reperfusion injury is associated with an inflammatory response, which leads to liver tissue injury, the release of reactive oxygen species (ROS), the induction of adhesion molecules, the secretion of cytokines and the activation of leukocytes^[43]. In addition, several immune cells, such as T cells, B cells, NK cells, NKT cells, and Kupffer cells,

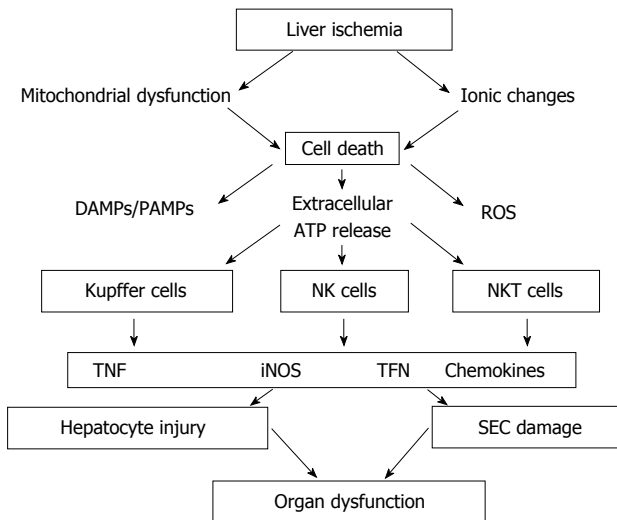


Figure 1 Simplified overview of the role of Kupffer cells, natural killer and natural killer T cells, including the humoral and cellular factors, involved in hepatocyte dysfunction and injury during hepatic ischemia-reperfusion injury. DAMPs: Damage associated molecular pattern; PAMPs: Pathogen-associated molecular pattern; ROS: Reactive oxygen species; TNF: Tumor necrosis factor; IFN: Interferon; iNOS: Inducible nitric oxide synthase; SEC: Sinusoidal endothelial cells.

are involved in hepatic ischemia-reperfusion injury^[44-51], which affect liver-specific cells, such as sinusoidal endothelial cells and hepatocytes^[52]. During cell injury and necrosis, danger-associated molecular patterns (DAMP) and, subsequently, pathogen-associated molecular patterns (PAMP) are released and trigger an immune response^[53,54]. Tissue ischemia leads to mitochondrial dysfunction, ATP depletion, and ionic changes within the cells, which promotes further cell damage and organ dysfunction (Figure 1)^[52].

HEPATIC NK CELLS

There is growing evidence that peripheral NK cells differ from hepatic NK cells with regard to function and differentiation; however, the exact mechanism of NK cell differentiation and maturation in the liver is not completely understood.

NK cells are the major lymphocyte population in the human liver and make up to 50% of the lymphocyte population. During liver disease, the number of NK cells in the liver changes possibly due to increased recruitment of NK cells to the liver^[9,55]. A diverse range of receptors expressed on the surface of NK cells allows them to recognize and rapidly respond to damaged or stressed cells. Furthermore, NK cells coordinate early events in the innate immune response to injury by rapidly producing cytokines and controlling cytotoxic activity.

Human NK cells in the blood can be distinguished from other T cells by the absence of CD3 and the presence of CD56^[56,57]. Furthermore, NK cells in the blood can be further differentiated into two major subsets: CD3⁻CD56^{dim}CD16⁺CD27⁻ (cytotoxic activity)

and CD3⁺CD56^{bright}CD16⁻CD27⁺ (cytokine producing)^[6]. Bone marrow-derived NK precursor cells undergo a complex maturation process, which determines their function and the expression of chemokine receptors and adhesion molecules^[6,58-60]. This determination is organ specific^[6,61]. Because NK cells recirculate between different organs, the maturation process is dynamic and not stationary^[58]. Adoptively transferred splenic NK cells change their phenotypic and functional markers after migrating to the liver, which suggests a modification of NK cells due to the hepatic microenvironment^[62]. In contrast to peripheral NK cells, hepatic NK cells lack CD16^[63,64], express higher numbers of granules, and express higher levels of TRAIL, perforin, and granzyme B^[65].

NK cells can potentially lyse dividing hepatocytes and/or other immune cells within the liver that contribute to the cytokine and chemokine microenvironment during regeneration and liver injury^[66,67]. NK cells actively eliminate susceptible targets through multiple, non-redundant mechanisms and recruit and amplify the inflammatory response^[68]. Because NK cells are closely linked to other immune cells, they are associated with Kupffer cells in the liver sinusoids, which suggests a complex interaction between these two cell types that involves cytokine and chemokine secretion^[69,70].

HEPATIC NATURAL KILLER T CELLS

Natural killer T (NKT) cells are a subset of regulatory T lymphocytes^[71]. In contrast to NK cells, NKT cells are found less frequently in the liver^[60], and their ultrastructure contains a low nuclear:cytoplasmic ratio and dense granules compared to NK cells^[72]. Therefore, NKT cells are less mature and have only a few organelles and mitochondria and short profiles of the rough endoplasmic reticulum^[72]. Compared to NK cells, the granules of NKT contain perforin, but are smaller in size and less frequently observed using electron microscopy^[73,74]. Interestingly, NKT cells have comparable functions with T cells, and NK cells and are able to secrete large amounts of cytokines^[72]. Similar to other immune cells, NKT cells are located within the liver sinusoids and are responsible for killing tumor cells, secretion of cytokines and elimination of toxins and pathogens^[60,75]. In addition, activated NKT cells are important for inducing liver injury^[76-78]. In contrast to NK cells, the number of NKT cells decreases during various experimental models, such as in leptin-deficient mice^[79], bacterial liver injury^[80], hepatotoxic liver injury^[81,82], liver steatosis^[83,84], and Concanavalin A-induced liver injury^[85]. However, following liver transplantation^[24], hepatic ischemia-reperfusion injury^[43,44], liver resection^[86-89] or stress^[90], the number of hepatic NKT cells increase. This change in cell number has been postulated to be due to activation-induced cell death, loss of specific NKT cell surface markers^[26,76,91,92], apoptosis^[93] or sympathetic activation^[89]. Flow cytometry analysis

of hepatic NKT cells shows that they are mostly CD4⁺CD8⁺ or CD4⁺CD8⁻^[94] and express the NK cell receptor-CD161 and the invariant TCR- α chain^[95]. NKT cells express IL-12 receptors and secrete and produce perforin and interferon (IFN)^[96] after stimulation, which are key mediators of cytotoxicity, inhibition of tumor angiogenesis and immune cell activation^[97,98]. Furthermore, NKT cells produce anti-inflammatory and anti-tumorigenic cytokines such as IL-13 and IL-4^[99-101].

KUPFFER CELLS

In 1876, von Kupffer first identified liver resident macrophages^[102]. These macrophages are colocalized with sinusoidal endothelial cells, Ito cells, and pit cells in the hepatic sinusoids^[103]. Kupffer cells are abundant in the liver and make up more than 50% of all resident macrophages in the human body and 15% of all hepatic cells^[104,105]. Depending on their location within the liver, the function, morphology and number of Kupffer cells changes^[103,106,107]. Interestingly, the intensity of immunohistochemical markers for Kupffer cells is heterogeneous. In general, the intensity of these markers decreases as the size of Kupffer cells decreases, which reflects a more immature phenotype that involves more scavenging and less inflammatory functions^[103,107]. The main function of hepatic macrophages is to clear the portal circulation from foreign materials and pathogens using phagocytosis^[103,108]. During this process, Kupffer cells release pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, TNF and IFN^[109].

SPECIFIC FUNCTION OF IMMUNE CELLS IN HEPATIC ISCHEMIA-REPERFUSION

Ischemia-reperfusion injury (IRI) significantly contributes to graft dysfunction after liver transplantation^[110]. Ischemia during the early phase of IRI leads to cell necrosis, which is associated with a release of danger signals that activate innate immune cells through signaling of TLR4, RAGE and TLR9 on Kupffer cells and through signaling of the CD154-CD40 pathway on neutrophils and CD4 Th1 effector T cells^[42]. This immune activation is further increased through the release of IFN from T cells, NKT and NK cells, which are stimulated by CD1d and CD39. Pro- and anti-inflammatory mediators further activate and recruit immune cells, which promotes or inhibits local inflammation^[42].

NK cells express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a potent inducer of hepatocyte cell death. In an experimental study, the effect of TRAIL expression on NK cells during hepatic IRI was investigated and showed that mice lacking TRAIL exhibited significantly higher liver injury, signs of necrosis, and neutrophil infiltration^[47]. The adoptive transfer of NK cells into immunodeficient

RAG2/common gamma null mice (lacking T, B and NK cells) revealed the specific role of NK cells during IRI and showed that the expression of TRAIL on NK cells is protective in a murine model of hepatic IRI^[47]. In another study, the effect of CD39, an ectonucleotidase hydrolyzing extracellular nucleotides, on NK cells was investigated and revealed that NK cells have an important influence on the extent of hepatic IRI. This effect was based on the modulation in IFN secretion, which was regulated by pericellular ATP levels and purinergic responses^[48]. Furthermore, it is postulated that liver resident NK cells are responsible for the innate immune response in the early phase of IRI through self/non-self-recognition^[49].

There are two types of NKT cells that have opposing roles during IRI to promote or protect against liver injury^[50]. During the early phase of IRI, NKT cells are promptly activated and release IFN^[50]. This activation is mediated by the interaction of CD1d antigen-presenting molecules, which are expressed on antigen-presenting cells in the liver and on hepatocytes containing self or foreign glycolipid antigens^[43,111]. NKT cells are then able to damage hepatocytes directly or through the secretion of IFN, which in turn activates Kupffer cells, neutrophils and hepatocytes^[43,111]. Knockout models with reduced NKT activity result in significantly reduced IRI^[43,44,111]. In addition, the recruitment of NK cells into the liver during IRI is dependent on the presence and activation of NKT cells^[50].

Hepatic hypoxia electron microscopy analysis revealed morphological changes in Kupffer cells that reflected cell activation^[112] and a release of cytokines and inflammatory mediators to attract neutrophils and produce reactive oxygen species^[113,114]. This activation is triggered by endogenous damage-associated and/or pathogen-associated molecular pattern (DAMP/PAMP) molecules, which are generated during cellular stress or cellular injury^[42]. During IRI, TLR4 on Kupffer cells is activated, which leads to hepatic injury^[115]. Activation of TLR4 enhances TNF secretion probably through an antigen independent pathway^[115,116] and is further associated with hepatocyte apoptosis^[117,118], CD4⁺ T cell recruitment to the liver^[119], and the release of endothelin-1, which results in circulatory disturbance and increased liver injury^[120,121]. Activation of the complement system is present during IRI^[122] and responsible for Kupffer cell-induced oxidant stress, the formation of reactive oxygen species and continuous neutrophil recruitment to the ischemic liver^[123]. Furthermore, inducible nitric oxide synthase (iNOS), which is produced by Kupffer cells and neutrophils early during hepatic IRI, leads to reduced capillary perfusion, increased liver injury and mortality^[124,125]. Activated Kupffer cells enhance alterations in hepatic microcirculation during IRI through the activation and production of oxygen free radicals^[126], TNF, MIP-2 and keratinocyte chemoattractant chemokine, which leads to increased liver injury^[127,128].

NK CELLS, NKT CELLS AND KUPFFER CELLS DURING GRAFT REJECTION AND TOLERANCE INDUCTION

It is postulated that the rejection of solid organ grafts is mainly mediated by allospecific T lymphocytes. These T lymphocytes recognize foreign MHC molecules that are located on donor tissue cells^[18,19]. However, it has been shown, that the depletion of CD8⁺ T cells does not prevent graft rejection and an alternative pathway of organ rejection has been postulated^[129,130]. Several studies using different experimental transplantation models have investigated the role of NK cells during graft rejection and demonstrated NK cell graft infiltration^[131-135]. Additionally, it has been shown that recipient-derived NK cells are located in the liver graft and produce IFN after liver transplantation^[24]. The depletion of NK cells or the decrease in IFN production leads to increased graft survival, therefore, NK cells are for graft rejection and survival^[24]. IFN, an immunoregulatory cytokine that is one of the main cytokines secreted of NK cells, has been shown to be important during both allograft rejection^[136-138] and tolerance induction^[139]. Studies investigating immunosuppression withdrawal demonstrated that NK cells play a role in tolerance induction^[140]. In addition, 13 genes that are highly expressed in NK cells, were found to be present in liver transplant recipients with graft tolerance, which further confirms that NK cells are involved in tolerance induction^[141]. Although this conflicting role of NK cells is still not fully understood, it might explain why donor NK cells are responsible for tolerance and recipient NK cells are responsible for rejection^[1]. In addition to cytokines, chemokines, such as CCL2, CCL3, CX3CL1 or CXCL10, attract and activate NK cells. Some of these chemokines are already present in the transplanted graft before NK cell infiltration is detectable^[142]. Specific analysis of NK cells in the rejected liver graft revealed that these NK cells produce high amounts of cytokines, granzyme B and highly express FasL^[135].

NKT cells are believed to be responsible for tolerance induction^[71]. Because activated NKT cells release pro- and anti-inflammatory cytokines, they have different functions in immune response^[143-145]. It has been further shown, that specific Va14 NKT cells are responsible for the development of tolerance towards transplanted antigens^[145].

As stated above, the main function of Kupffer cells is to kill and engulf microorganisms and pathogens, secrete cytokines and effect antigen presentation^[146,147]. Additionally, it has been shown, that Kupffer cells are able to induce T cell apoptosis and therefore play an important role during graft tolerance^[148]. After liver transplantation Kupffer cells act as antigen-presenting cells by increasing the expression of MHC class II^[149,150] and identifying and interacting with recipient T cells migrating to the liver, which leads to T cell apoptosis

through the Fas/FasL pathway^[109]. In a study in rats, pretreatment of the recipients with Kupffer cells before liver transplantation lead to decreased liver injury, reduced cytokine levels and reduced apoptosis. The authors concluded that this lead to increased immune tolerance and improved graft survival^[148]. As mentioned above, Kupffer cells secrete varying amounts of cytokines, such as TNF, which in high levels can lead to hepatocyte apoptosis but in physiological levels is associated with a resistance of hepatocytes to apoptosis^[151]. Therefore, further studies are necessary to elucidate the contrasting roles of Kupffer cells in the induction of immune tolerance following liver transplantation.

CONCLUSION

Several specific immune reactions that involve NK, NKT and Kupffer cells are responsible for the short- and long-term outcomes of liver transplantation. This review demonstrates that many immune cells and mediators as well as molecular signaling cascades participate in the process of liver transplantation tolerance. Despite intense research within the field of ischemia-reperfusion injury, there are still many pathophysiological and immunological mechanisms involved in tolerance induction and graft rejection that still need to be elucidated.

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2016 Liver Transplantation: Global view

Doppler ultrasonography in living donor liver transplantation recipients: Intra- and post-operative vascular complications

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Abstract

Living-donor liver transplantation has provided a

solution to the severe lack of cadaver grafts for the replacement of liver afflicted with end-stage cirrhosis, fulminant disease, or inborn errors of metabolism. Vascular complications remain the most serious complications and a common cause for graft failure after hepatic transplantation. Doppler ultrasound remains the primary radiological imaging modality for the diagnosis of such complications. This article presents a brief review of intra- and post-operative living donor liver transplantation anatomy and a synopsis of the role of ultrasonography and color Doppler in evaluating the graft vascular haemodynamics both during surgery and post-operatively in accurately defining the early vascular complications. Intra-operative ultrasonography of the liver graft provides the surgeon with useful real-time diagnostic and staging information that may result in an alteration in the planned surgical approach and corrections of surgical complications during the procedure of vascular anastomoses. The relevant intra-operative anatomy and the spectrum of normal and abnormal findings are described. Ultrasonography and color Doppler also provides the clinicians and surgeons early post-operative potential developmental complications that may occur during hospital stay. Early detection and thus early problem solving can make the difference between graft survival and failure.

Key words: Doppler; Ultrasound; Living donor; Liver transplantation; Intraoperative; Postoperative; Vascular; Complications

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Core tip: In this article we focus on the role of intra- and post-operative Doppler ultrasonography in the early detection of potential vascular complications in living-donor liver transplantation, in addition to monitoring the surgical and interventional vascular therapeutic procedures.

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INTRODUCTION

Living donor liver transplantation (LDLT) is an established therapeutic modality for adults and paediatrics with end-stage liver diseases and congenital hepatobiliary diseases, especially in countries where deceased donors for liver transplantation (LT) are not available and where the waiting lists for orthotopic liver transplantation are too long for critically ill patients with end-stage liver disease.

Vascular complications (VCs) remain the most serious complications and a common cause for graft failure after LT^[1]. The overall incidence of VCs is higher in LDLT than in deceased donor liver transplantation (DDLT) due to the complex nature of the vascular anastomoses. The reported incidence of vascular complications after LT in adults varies widely among transplant centres, ranging between 8%-15%. However, this rate can be as high as 20%, especially in cases such as split liver transplantation or LDLT^[2-5]. Higher incidence has been reported in paediatric patients because of their smaller vessels in addition to the short vascular pedicles that are available for reconstruction^[6].

Intraoperative ultrasound (IOUS), a non-invasive test that allows real-time and quantitative evaluation of the graft vasculature, has been considered an integral component of the recipient surgery. Its use in early detection and, thus, in the immediate surgical correction of intra-operative (IO) VCs ensures adequate graft perfusion after revascularization. The sonographer should be able to provide the surgeon with sufficient data regarding the morphology and integrity of the different surgical anastomoses. Furthermore, with the aid of different Doppler flow measurements, the hemodynamic changes can be described and interpreted. The decision to perform vascular repair of the anastomoses is multidisciplinary and depends on a variety of factors rather than solely the Doppler ultrasound (DU) findings.

DU is the modality of choice for PO recipient surveillance as it is non-invasive, portable and provides rapid, comprehensive and accurate evaluation of the entire hepatic vasculature. Knowledge of the immediate and early physiological graft hemodynamics after graft perfusion and early identification of VCs are essential for improving graft and patient survival^[7].

In this article, we review the role of intra- and post-operative DU in the evaluation of the recipient surgery during LDLT, with descriptions of the surgical background, the IOUS technique, normal DU appearance of different

vascular anastomoses, the spectrum of normal and abnormal graft haemodynamics and potential VCs.

SURGICAL BACKGROUND

In an adult recipient, a right lobe graft that is drained by the right hepatic vein (RHV) is obtained by transecting the liver on the right side of the middle hepatic vein (MHV). The resection typically includes the entire right lobe, RHV, right portal vein, right hepatic artery and right bile duct. The middle hepatic artery (segment IV artery) and MHV are preserved for survival of the medial segment of the donor liver and are critical for avoiding venous complications in the donor^[8,9] (Figure 1A).

Implantation of the graft starts with hepatic vein (HV) reconstruction, followed by portal vein (PV) and then hepatic artery (HA) reconstruction. The RHV is either reconstructed in an end-to-end fashion to the stump of the RHV in the recipient or anastomosed in an end-to-side fashion directly to the inferior vena cava (IVC). Reconstruction of large-calibre MHV tributaries, such as HV draining segment V (V5) and segment VIII (V8), is often essential to avoid congestion of the segment drained by this tributary^[10]. Various reconstruction techniques have been reported using autologous vein grafts such as the greater saphenous vein, Left PV (LPV) or the para-umbilical vein. Moreover, some techniques have been reported using cryo-preserved veins or arteries^[11]. Additionally, synthetic grafts are widely used in many centres for venous reconstruction. The large-calibre inferior RHV might also require reconstruction, usually in an end-to-side fashion to the IVC^[12,13].

Modalities for PV reconstruction are chosen according to the diameter, size mismatch, wall status, and length of the recipient PV. Reconstruction is performed by end-to-end anastomosis between the graft right PV and recipient main PV. When PV reconstruction is impossible, for example, due to pre-operative PV thrombosis (PVT), a venous jump or interposition conduit must be obtained^[14].

HA reconstruction carries its own challenges due to the small vessel diameter, the deeply seated vessels, the short stump, and the moving field. Furthermore, tachycardia and tachypnoea can add to the difficulties^[15]. The selection of the recipient artery is critical for successful anastomosis. The artery is chosen according to the patency, size match, length, direction, and very importantly, the upfront blood flow^[16]. One common problem is the discrepancy in size between the graft and recipient arteries. Different techniques have been described for an anastomosis in such cases, including oblique cut, fish mouth method, funnelization and end-to-side techniques^[17]. In cases of grafts with double arteries, a single opening may be created or two separate arterial anastomoses may be performed^[18,19]. A single anastomosis may also be performed using the larger artery, provided the other

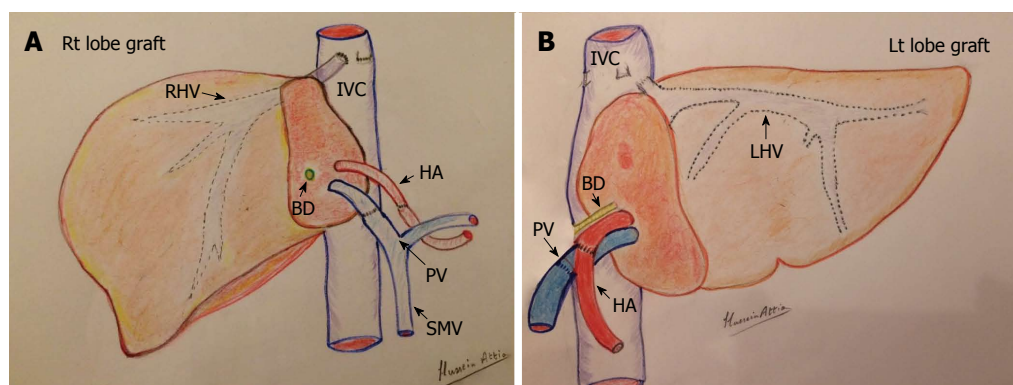


Figure 1 Diagram of the surgical technique of graft implantation in living donor liver transplantation. A: Right lobe graft in an adult patient; B: Left lobe lateral segment in a child. HA: Hepatic artery; PV: Portal vein; IVC: Inferior vena cava; BD: Bile duct; SMV: Superior mesenteric vein; RHV: Right hepatic vein; LHV: Left hepatic vein.

artery presents good backflow^[15].

For most paediatric recipients, the donor liver graft is the left lobe or lateral segment (Figure 1B). The graft left HA and left PV are anastomosed end-to-end to the appropriate HA and PV of the recipient. The left HV is anastomosed in an end-to-end fashion with the LHV stump of the recipient^[16].

HEPATIC INTRA-OPERATIVE ULTRASONOGRAPHY OF RECIPIENTS IN LDLT

IOUS has become an integral part of LT surgery that provides the surgeon with real-time information regarding the integrity of vascular anastomoses and graft hemodynamics. Reconstructive procedures are monitored by DU guidance until optimum flow is established. This technique reduces post-transplant vascular complications that might necessitate re-transplantation^[20].

Before performing IOUS, the sonographer should revise the pre-operative imaging, including the DU data and the multi-detector computed tomography angiography of both the recipient and the donor to verify the normal and variant vascular branching anatomy and the liver haemodynamics prior to transplantation. The surgical technique is discussed with the surgeons regarding the type of anastomoses, size mismatch, usage of interposition grafts and the presence of any technical problems during the surgical reconstruction.

TECHNICAL CONSIDERATIONS

IOUS is performed before starting the biliary anastomosis. Scanning may be delayed for few minutes after graft perfusion to allow the HA to recover from spasticity. Linear array, 7-8 MHz transducers are most frequently used^[21]. T- or V-shaped transducers are preferred to facilitate scanning the liver surface and the vascular pedicle. Some machines are equipped

with remote control units that facilitate the scanning technique. The upper abdominal cavity is filled with warm saline as an acoustic medium for the US beam (Figure 2). The operating table can be tilted to the left side to avoid fluid spillage. During examination of the extrahepatic vessels (PV and HA), the whole vessel should be well immersed in the fluid, at a certain distance from the transducer. Assistance may be required from the surgeon to unfold vascular kinks and to move vascular clamps away from the US field. During the examination of the intrahepatic vessels (post-anastomotic and segmental branches), the transducer can be applied directly to the surface or the cut surface of the graft, very gently to avoid injuring the potentially bleeding cut surface.

HV evaluation

The probe can be tilted in the axial plane, cranially guided by the IVC, with gradual angulation towards the graft cut surface. In the b-mode, the vein is unfolded to be in line with the anastomosis. The anastomosis is usually elliptical in shape and in this axial oblique plane, only the antro-posterior diameter of the anastomosis can be visualized. The cranio-caudal diameter cannot be demonstrated using this technique; it is necessary to scan the anastomosis cranially and caudally until the widest point in the anastomosis is visualized (Figure 3). The anastomotic/intra-hepatic HV velocity ratio can then be calculated. Reconstructed segment V or segment VIII grafts and an accessory right hepatic vein anastomosed to the IVC can be scanned in the same manner (Figure 4). The use of synthetic grafts usually hinders the US beam; in such a condition, assessment of the intrahepatic flow is usually sufficient to ensure patency of the graft (Figure 5).

PV evaluation

The PV is examined at the graft hilum (Figure 6); the probe is angulated to demonstrate the entire length of the recipient PV down to the confluence of the splenic and superior mesenteric veins for detection of thrombi. The size mismatch between the recipient

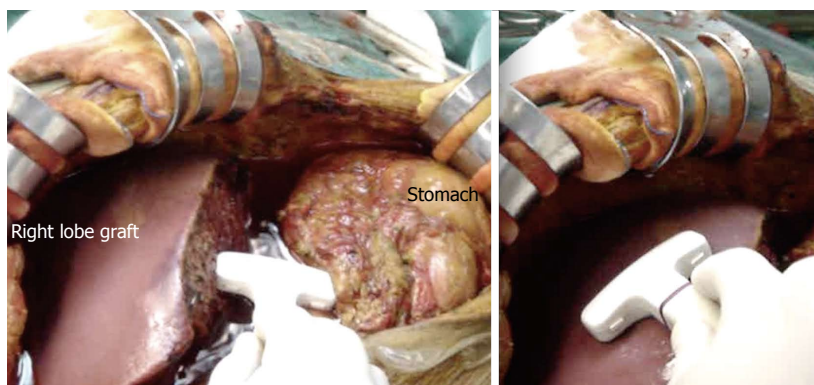


Figure 2 Photography showing the technique of intraoperative ultrasound. The upper abdominal cavity is filled with warm saline and a T-shaped 7-9 MHz linear transducer is applied at the graft hilum and graft surface.

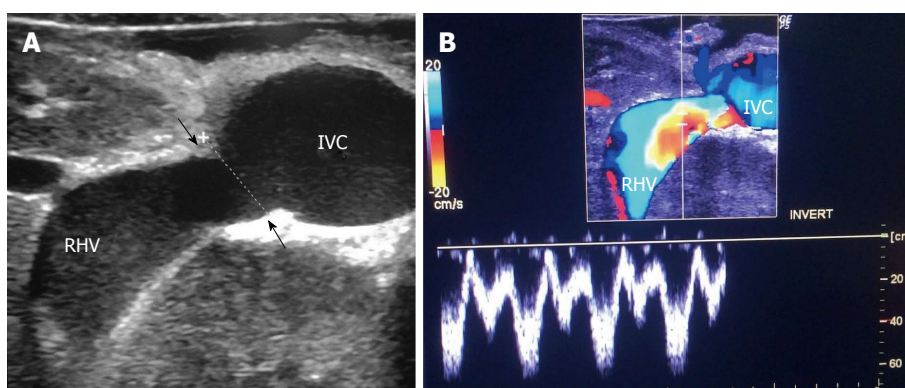


Figure 3 Intra-operative ultrasound evaluation of right hepatic vein anastomosis. A: B-mode US image showing RHV with end-to-side anastomosis with the IVC; B: Color Doppler image showing normal triphasic waveform of the RHV. RHV: Right hepatic vein; IVC: Inferior vena cava.

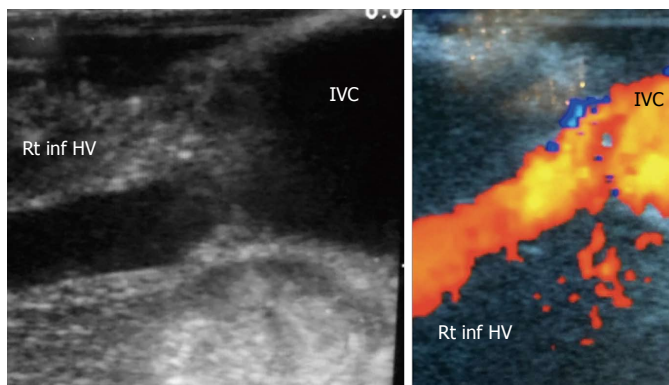


Figure 4 Intra-operative ultrasound evaluation of right inferior hepatic vein anastomosis. B-mode and color US image showing the anastomosis of accessory right inferior HV, end-to-side with the IVC. Rt inf HV: Right inferior hepatic vein; IVC: Inferior vena cava.

and donor veins and the diameter of the anastomosis are measured, after which the anastomotic/pre-anastomotic velocity ratio can be calculated. Finally, examination of the intrahepatic segmental branches is important to ensure adequate graft perfusion.

HA evaluation

The HA can be unfolded with the aid of the surgeon, and its patency and the presence of any thrombi, dissecting flaps or anastomotic strictures can be

evaluated. The site of anastomosis can be visualized as two echogenic dots on the wall (Figure 7). Measurements of peak systolic velocity (PSV), end diastolic velocity (EDV) and resistivity index (RI) are taken after proper angle correction in the pre-anastomotic, anastomotic and post-anastomotic segments. The velocity ratio (anastomotic/pre-anastomotic) can then be calculated. Examination of the intrahepatic arterial waveform is of utmost importance as a proper intrahepatic arterial flow with a

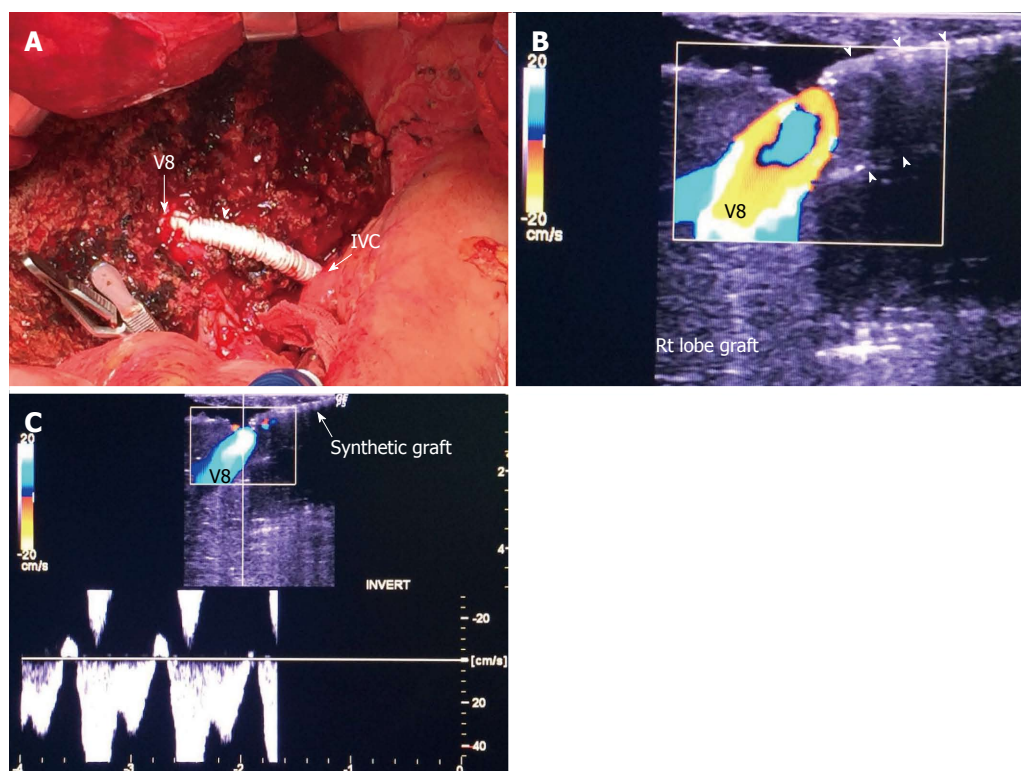


Figure 5 Intra-operative ultrasound evaluation of segment VIII synthetic graft. A: Photography showing the synthetic graft (arrow head) between V8 and IVC; B: Color image showing patent segment VIII vein and absence of color flow in the synthetic graft (arrow heads); C: Color Doppler image showing normal triphasic waveform in segment VIII vein, confirming synthetic graft patency. V8: Segment VIII hepatic vein; IVC: Inferior vena cava.

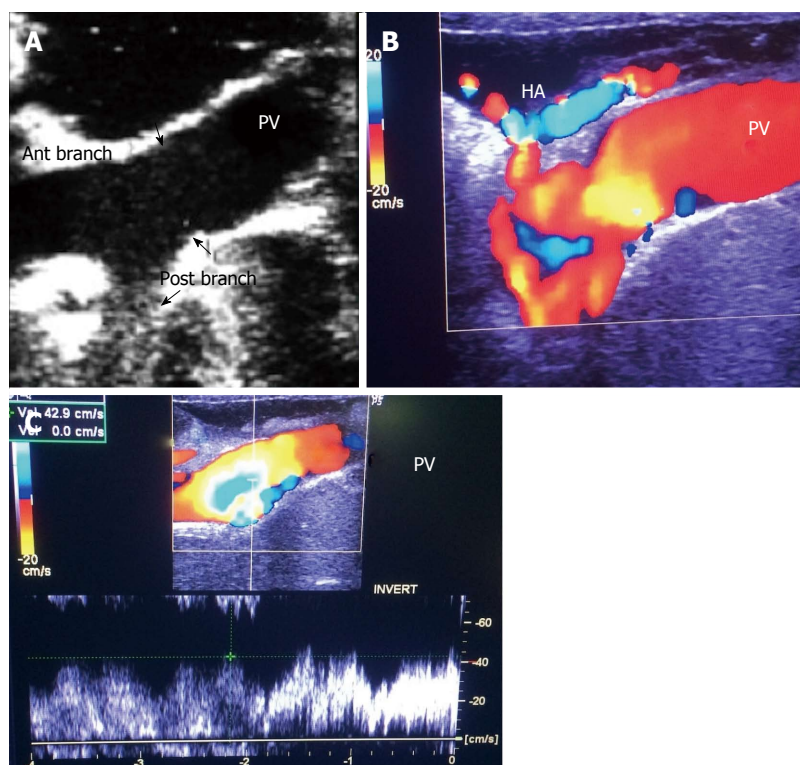


Figure 6 Intra-operative ultrasound evaluation of portal vein anastomosis. A: B-mode ultrasound image showing the extrahepatic portal vein and intrahepatic bifurcation. The suture line appears as echogenic shadows at the site of anastomosis (arrows); B: Color image showing homogeneous color saturation in the PV with mild aliasing at site of anastomosis and at the PV bifurcation; C: Color Doppler image showing antegrade normal pulsatile flow in the PV. PV: Portal vein; HA: Hepatic artery.

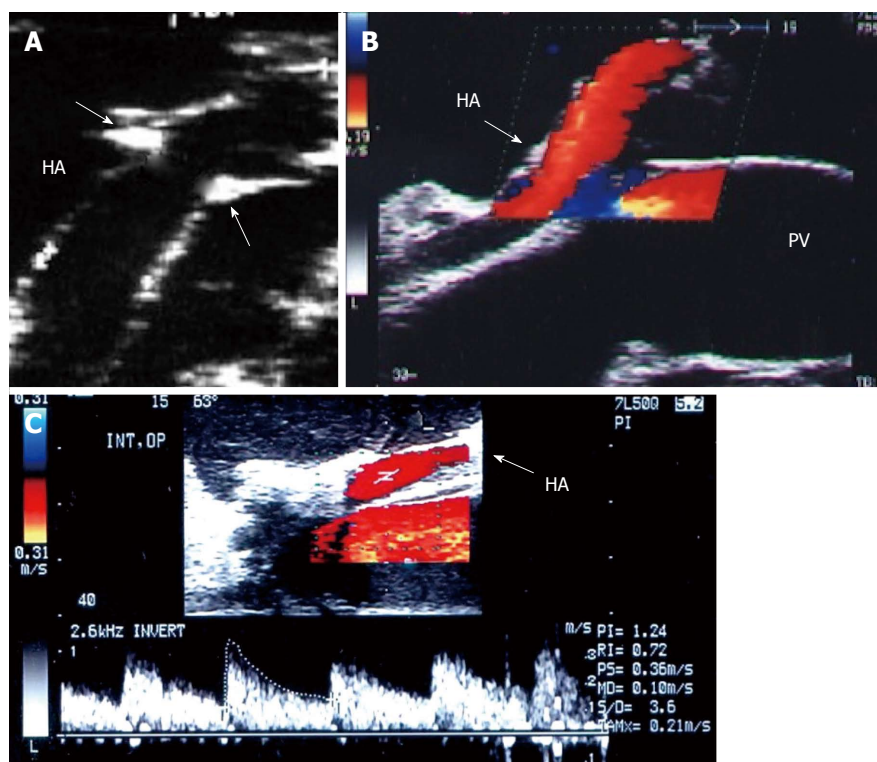


Figure 7 Intra-operative ultrasound evaluation of hepatic artery anastomosis. A: B-mode ultrasound image showing the HA anastomosis, appearing as an echogenic shadows at the site of anastomosis (arrows); B: Color image showing homogeneous color saturation in the HA (arrow); C: Color Doppler image showing normal biphasic waveform of the HA (arrow). PV: Portal vein; HA: Hepatic artery.

good arterial spectral waveform often signifies a proper anastomosis.

In paediatric patients, the scanning technique is different; the PV and HA anastomoses lie beneath the left lobe graft in the neutral position. The oblique entry of the left PV and left HA into the graft (C-shaped) creates technical difficulties in unfolding the vessel to evaluate the anastomoses (Figure 8). The surgeon can retract the liver gently upwards to allow scanning however, this retraction places the vessel and anastomoses under tension that may alter the DU measurements. One option is to perform the scanning from the surface of the graft while in the neutral position. This technique may require low- frequency transducers, and probing should be performed gently to avoid injury to the graft surface during probe angulations.

POST-OPERATIVE ULTRASONOGRAPHY OF RECIPIENTS IN LDLT

DU is often employed in the initial recipient work-up post-LDLT. DU is the imaging technique of choice to assess early and late surgical complications as it provides rapid, comprehensive and accurate evaluation of the entire hepatic vasculature and graft parenchymal abnormalities (Figure 9).

The timing and frequency of postoperative US screening ranges from a single examination on the postoperative day or every 12 h to daily or alternate-

day examinations for 14 d, or daily until discharge^[22], which is our practice.

DU is performed at the patient's bedside with the patient in the supine or semi-sitting position using low-frequency (4-5 MHz) curvilinear transducers. Sterilized gel is preferred to avoid wound contamination. In addition to DU evaluation of the graft perfusion, ultrasound of the abdomen is performed for the evaluation of the hepatic parenchyma, peri-hepatic fluid collection and ascites^[7].

Scanning of the vascular anastomoses is usually performed in the anterior axillary intercostal window. The HA and PV are examined extrahepatically, the DU parameters across the anastomoses are recorded, and their segmental intrahepatic branches are then examined to evaluate the graft perfusion. The anterior segmental branches are usually chosen as they are nearer to the probe and are easily assessed. The main HV and accessory veins are evaluated intrahepatically. If definite diagnosis of a VC is established by DU, interventional radiological treatment or surgery can be performed. In equivocal cases, cross-sectional imaging by MDCT angiography is performed prior to intervention.

NORMAL DOPPLER ULTRASOUND OF A HEALTHY GRAFT

The HV in a healthy graft displays a similar spectral waveform as a healthy native liver, typically a

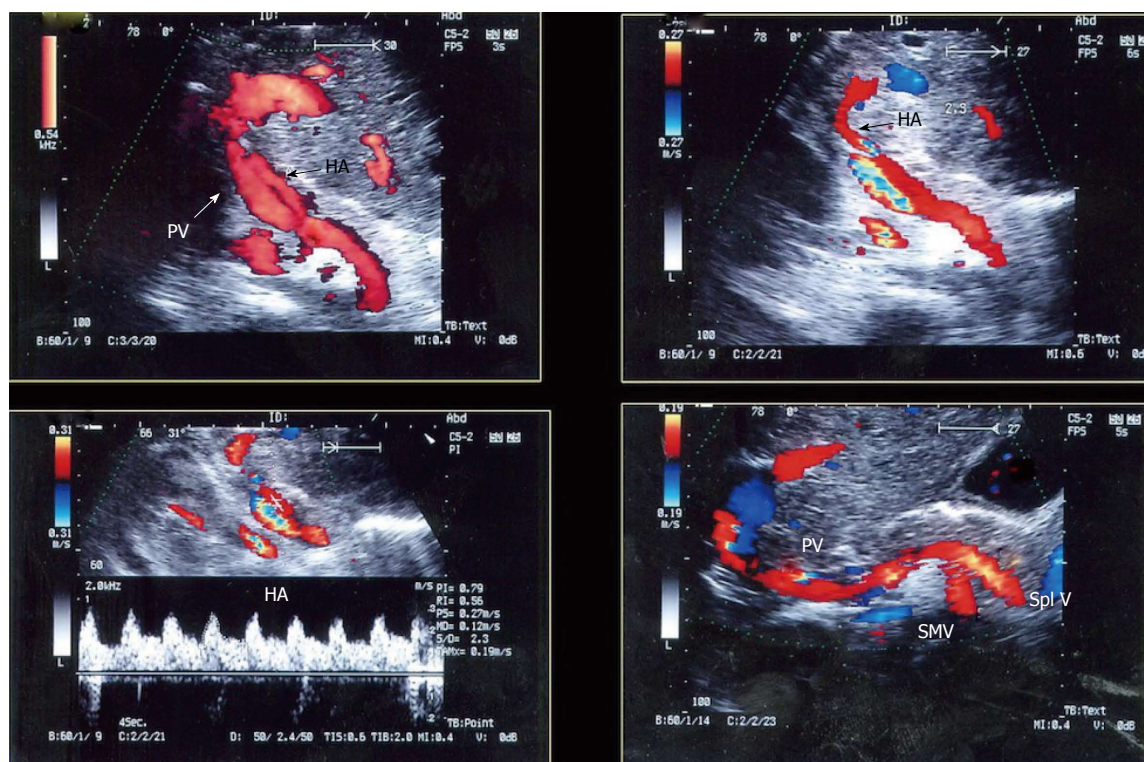


Figure 8 Intra-operative ultrasound evaluation of the portal vein and hepatic artery in left lobe transplants. Color Doppler images showing the PV and HA anastomoses. Note the C-shaped entry of the vessels at the graft hilum. PV: Portal vein; HA: Hepatic artery.

triphasic, hepato-fugal waveform corresponding to the cardiac cycle, with two antegrade major peaks followed by a small retrograde component in the early ventricular systolic phase (Figures 3B and 9C). Changes in the HV spectrum can be noted with phases of respiration, fluid status, cardiac dysfunction or abnormal anastomosis^[23]. Elevation of the HV velocity at the anastomotic site (< 3 -fold) compared to the intrahepatic velocity can be accepted in case the phasic pattern is preserved and the flow velocities are satisfactory (Figure 10).

The PV has a wavy venous spectral waveform with respiratory variant pattern changes and has been described in earlier reports as being non-pulsatile or continuous (parabolic). With the recent interest in analysis of the portal vein waveform, a more precise description has emerged concerning the undulant fluctuation of flow; hence, different authors have used the term pulsatility^[24]. Average blood flow velocity in the main PV is approximately (12-30 cm/s) in healthy adults^[25]. However, in LDLT the normal range of PV velocities has not been agreed upon but is usually more than 30 cm/s as the donor PV component calibre, the right main branch, is usually smaller than recipient's main PV component in right lobe grafts. Additionally, the recipient large portal flow volume is directed to a partial (split liberal) instead of a whole native liver size. These 2 factors contribute to the higher velocity values measured immediately after portal anastomoses (Figure 11). In left lobe grafts the recipient PV is usually smaller than the graft PV and

the elevation in the anastomotic and post anastomotic velocities are not usually expected (Figure 12).

In healthy individuals, the spectral Doppler of the HA presents a biphasic waveform pattern; both systolic and diastolic waves are observed above the baseline, and no waves are detected below it (Figures 7 and 9B). The RI (PSV- EDV/ PSV), which allows a semi-quantitative estimation of the resistance to arterial flow into the liver, is the most commonly used Doppler parameter in hepatic artery evaluation. Under physiologic conditions, the HA flow shows low-resistance flow with antegrade flow throughout diastole because of the broad arterio-portal and arterio-sinus connections in the hepatic micro-vascular system. Its normal value in both healthy individuals and those with transplants, ranges from 0.55 to 0.8^[26,27].

In cirrhotic patients with portal hypertension, the hyper-dynamic splanchnic circulation may results in hypertrophy of the HA with increased dimension^[28-30]. This explains the frequent finding of HA size mismatch between the recipient and graft HA especially in right lobe transplants (Figure 13). Variations in the HA flow may occur normally due to anastomotic caliber discrepancies, central arterial pressure differences and the frequent occurrence of transient arterial spasm.

EARLY GRAFT HAEMODYNAMICS AFTER LDLT

Early detection of haemodynamic abnormalities

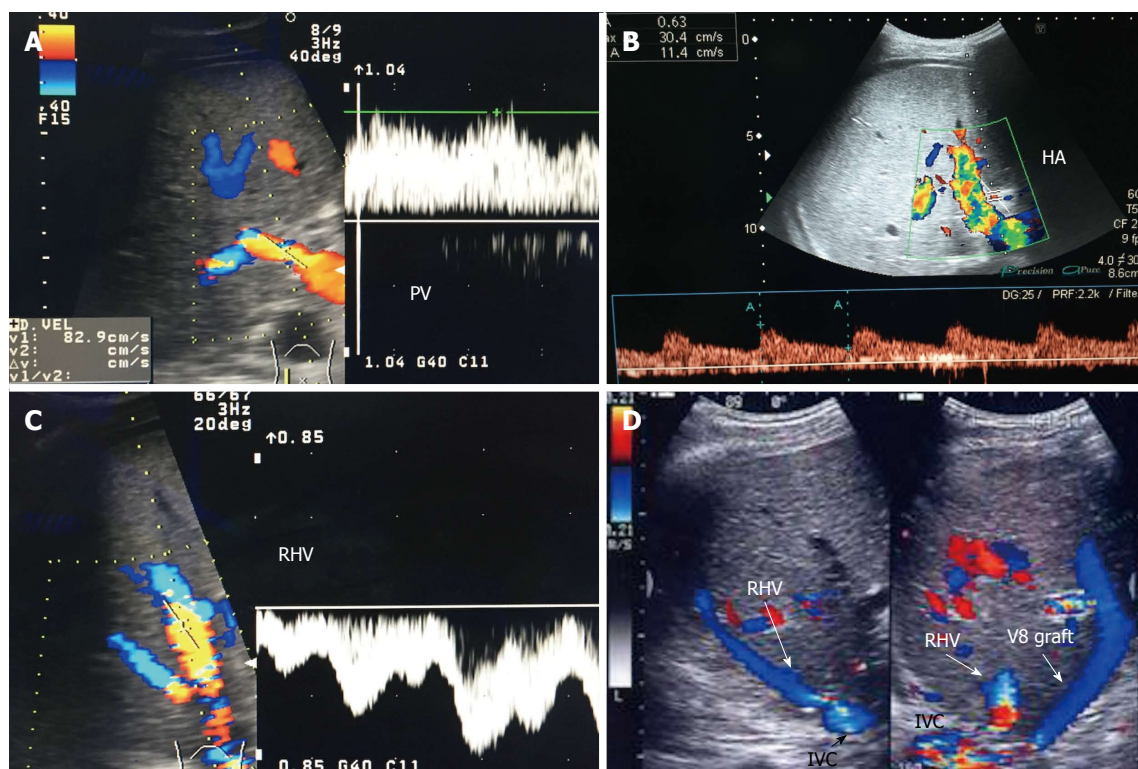


Figure 9 Post-operative Doppler ultrasound evaluation of the graft vasculature. A: Color Doppler image demonstrating normal petal, slightly turbulent, high flow in the PV (83 cm/s); B: Color Doppler image showing normal biphasic flow in the HA with normal RI = 0.63; C: Color Doppler image showing normal triphasic flow in the RHV; D: Color US image showing patent autologous V8 graft. PV: Portal vein; HA: Hepatic artery; IVC: Inferior vena cava; RHV: Right hepatic vein; V8: Segment VIII vein.

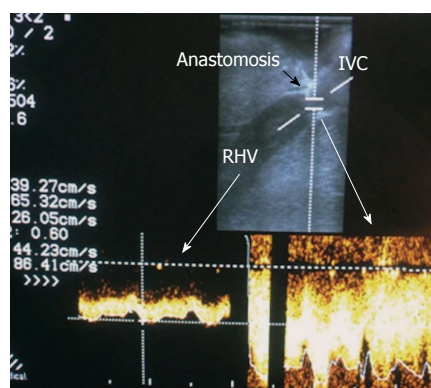


Figure 10 Normal right hepatic vein anastomosis. Intra-operative color Doppler image demonstrating normal augmentation of the RHV flow at the site of anastomosis (2-fold) compared to the intrahepatic flow, with preserved phasic flow pattern. IVC: Inferior vena cava; RHV: Right hepatic vein.

after LT requires knowledge of the normal or physiological changes that accompany the graft perfusion immediately and in the early PO period. The haemodynamics of the transplanted graft differ from those of a native liver as they depend on the inflow, represented by the PV and HA, and outflow through a single HV^[15]. Analysis of DU findings may be confusing intra-operatively and in the early PO period due to complex vascular anastomoses, donor-recipient vascular size mismatch and reduced graft volume.

For patients with cirrhosis, immediately after LT,

the mechanical component of portal hypertension is relieved by the healthy graft but without immediate restoration of the systemic or the splanchnic circulation to normal levels^[31]. The splanchnic circulation shows rapid and potentially reversible changes in the portal and arterial perfusion that may not be clinically significant^[32-34]. However, the changes in the portal and arterial parameters by DU are still under debate.

After orthotopic liver transplantation (OLT), a high perfusion state develops that is predominantly portal, with increases in the PV flow and velocities and the hepatic arterial resistance. In LDLT, the haemodynamic changes are much more pronounced than those occurring in DDLT, with a higher perfusion state and increases in the portal venous flow and velocities^[35-37]. The increased PV velocity has been attributed to the persistence of the hyperkinetic haemodynamic splanchnic circulation in patients with cirrhosis and portal hypertension, reduction in the liver vasculature and small PV anastomotic stoma that could induce elevations in PV resistance and pressure, effects of the loss of sympathetic hepatic innervation or elevated cardiac output^[31,33,34,36,38,39]. A wide range of PV velocities (15-400 cm/s) have been reported in the immediate post-OLT period in patients without vascular complications^[40].

High-resistance arterial flow is frequently detected in patients with a normal hepatic artery. This flow returns to normal in few days and has not been

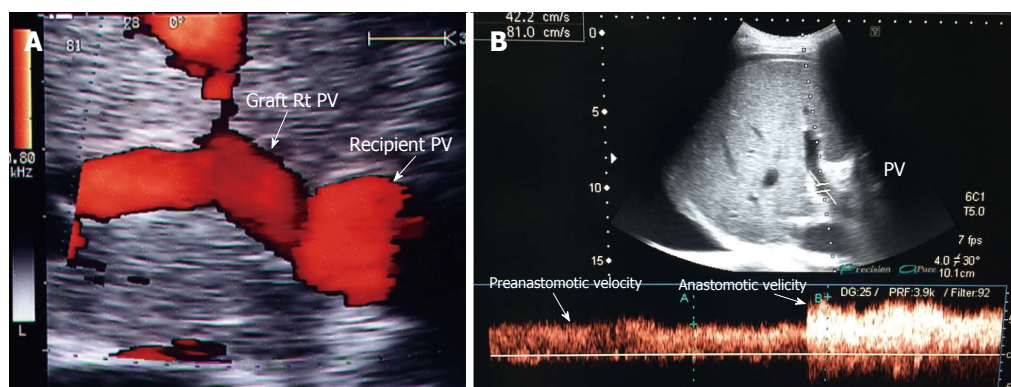


Figure 11 Portal vein size mismatch in right lobe grafts. A: Post-operative color ultrasound image showing size mismatch between the recipient and donor PVs, a frequent finding in right lobe living donor liver transplantation; B: Color Doppler image showing anastomotic augmentation of the PV velocity (2-fold) compared to the pre-anastomotic velocity (accepted finding in the presence of size mismatch). PV: Portal vein.

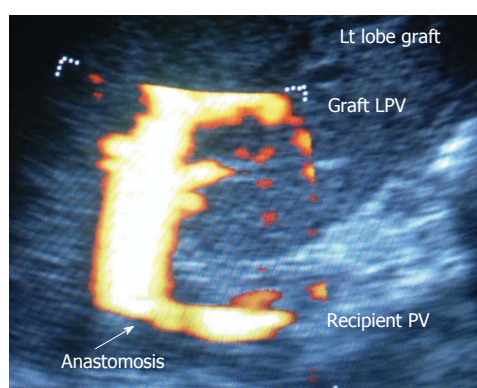


Figure 12 Portal vein size mismatch in left lobe grafts. Color ultrasound image showing significant size mismatch between the small recipient PV and the graft left PV in pediatric living donor liver transplantation. PV: Portal vein; LPV: Left portal vein.

associated with deterioration in the clinical course or decreased graft and patient survival^[15,41,42]. The elevated RI has been attributed to the regulatory mechanisms and the hepatic arterial buffer response that induces HA vasoconstriction in response to portal hyper-perfusion and produces a high resistive index with poor arterial perfusion^[43,44]; it has been demonstrated by temporary clamping of the portal vein that resulted in improvement of the hepatic arterial flow^[15]. Other theories related the early and transient elevation of the HA RI after DDLT to the older donor graft, prolonged periods of cold ischaemia and preservation injury, graft steatosis and chronic cholestatic disease^[43,45,46].

INTRA- AND POST-OPERATIVE VASCULAR COMPLICATIONS IN LDLT

Hepatic vein complications

Different techniques and innovations have been proposed for reconstruction of the main graft HV and large accessory veins and tributaries. Ensuring the integrity of those anastomoses by DU is crucial

because there is no collateral route for blood to exit the liver; failure to establish proper venous drainage may result in congestion and life-threatening graft dysfunction^[10,47,48]. HV complications after LT are relatively uncommon, with reported incidence ranging from 1%-6%; this incidence is 2 times higher in LDLT^[4,49-52].

HV outflow obstruction is a general term referring to obstruction of the HV that may occur intra-operatively due to surgical error or graft torsion and post-operatively due to compression/twisting of the anastomosis resulting from graft regeneration or intimal hyperplasia and fibrosis at the anastomosis. Clinical signs are usually nonspecific and include congestion of the liver parenchyma with abnormal laboratory values, hepatomegaly, ascites, and pleural effusions^[53].

Diagnostic criteria of HV stenosis (HVS) using DU include the morphological appearance of the anastomosis by b-mode US, alteration of the Doppler waveform, velocity measurements and secondary compromise in the portal flow (Figures 14 and 15). HVS appears on b-mode US as an abrupt shouldering (waist) of the anastomosis or gradual tapering (beak-like)^[54]. Loss of the triphasic pattern of the HV and absence of the retrograde flow are believed to reflect increased stiffness of the liver parenchyma around the HVs and had a 98.4% negative predicted value for venous obstruction^[55]. In contrast, a persistent triphasic wave pattern on Doppler ultrasound images can exclude the possibility of substantial stenosis^[53].

However, monophasic waveforms alone are not specific for HVS and associated dampening of the flow velocities usually accompany the loss of various components of the triphasic pattern^[54,56]. Low peak HV velocity (as low as 10-11 cm/s) has been reported in previous studies^[57,58]. In addition, dilatation of the hepatic vein and secondary compromise of the portal flow with extreme decrease in PV velocity < 14 cm/s have been reported^[58]. We believe that measuring the velocity gradient (anastomotic/intrahepatic) is

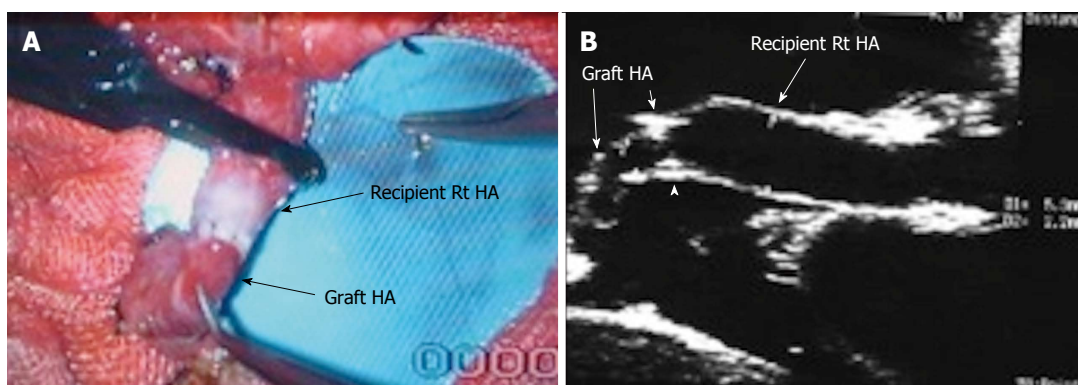


Figure 13 Hepatic artery size mismatch. A: Photograph showing size mismatch between the recipient and donor HAs during right lobe living donor liver transplantation; B: B-mode US image showing hypertrophied recipient HA and small graft HA with good anastomosis (arrow heads). HA: Hepatic artery.

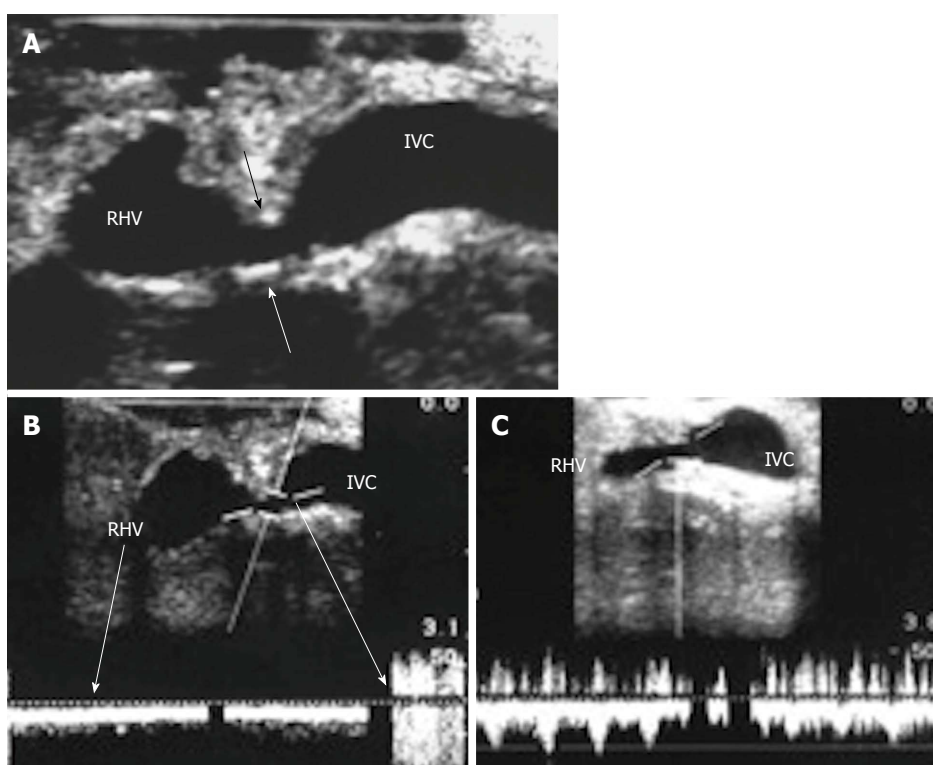


Figure 14 Intra-operative hepatic vein stenosis. A: Intra-operative B-mode US image showing tight stricture along the RHV anastomosis with the IVC (arrows); B: Pulsed Doppler image showing dampened intra-hepatic venous spectral flow, loss of the normal venous phasicity and anastomotic jet (> 3-fold) compared to the intrahepatic flow; C: Pulsed Doppler image after surgical revision of the RHV anastomosis demonstrating improvement of the flow with regaining the triphasic pattern respecting the cardiac cycle. RHV: Right hepatic vein; IVC: Inferior vena cava.

essential for grading the degree of venous outflow compromise and we consider anastomotic jets > 3-fold compared to the intrahepatic velocities to be significant (Figure 14B). In equivocal cases, measuring the pressure gradient intra-operatively or during PO radiological interventional procedures can help distinguish haemodynamically significant lesions from pseudo-stenosis. A pressure gradient between the HV and the IVC > 10 mmHg was considered substantial HVS^[53]. In addition IO graft discoloration and increased stiffness and PO clinical signs would aid in establishing the diagnosis.

Because revision of the HV anastomosis carries its own challenges and might require clamping of the vascular pedicle, it is important to rule out torsion before rushing to the diagnosis of surgical anastomotic stricture based on the DU findings. Repositioning the graft in either direction and repeating the DU can be performed several times until a proper flow and waveform are established; hepatopexy can then be performed in the optimum position.

For treatment of PO HVS, percutaneous trans-jugular or trans-hepatic balloon angioplasty and stent placement are the modalities of choice, with adequate



Figure 15 Post-operative hepatic vein stenosis in a patient with persistent ascites and graft dysfunction, 2 mo post-living donor liver transplantation. A: Post-operative B-mode US image showing tight stricture along the RHV anastomosis with the IVC (arrows); B: Pulsed Doppler image showing dampened hepatic venous spectral flow, loss of normal hepatic venous phasicity and anastomotic jet (5-fold) compared to the intrahepatic flow; C: MDCT angiography, coronal reconstructed image showing RHV anastomotic stricture (arrows); D: Digital subtraction trans-hepatic venography showing significant RHV anastomotic stricture (arrows). The pressure gradient was > 10 mmHg across the anastomosis. Balloon angioplasty and stent placement were performed. RHV: Right hepatic vein; IVC: Inferior vena cava.

clinical and technical success rates^[51,52,59,60].

HV thrombosis is a rare complication in LT that has been reported in a few PO cases with clinical manifestations of HV outflow obstruction and is considered a sequel of untreated HVS^[61-63]. Acute thrombosis within the segmental ligated HVs (V5 and V8) appears on DU as hypo-echoic with absent flow on color Doppler mapping and sometimes shows a very damped (to-and-fro flow) within the vein. On serial PO examination, the echogenicity of the thrombus gradually increases and is associated with echogenic geographically congested areas along the vein distribution, that usually resolve within 1-2 wk PO. If the ligated branch is large, large areas of congestion results that might cause graft dysfunction (Figure 16). Whereas thrombosis in the main HV is a very rare and dreadful complication that necessitates immediate intervention, we experienced one case of fatal IO massive thrombosis of the main RHV and one patient with a small adherent thrombus that was managed conservatively (Figures 17 and 18).

Portal vein complications

PV complications following LT are relatively uncommon, occurring in 1%-3% in most reports (with an incidence reaching 13%), and are associated with high morbidity and graft loss^[1,64-69]. They are more common with split

liver and LDLT^[65,70]. Risk factors include significant size mismatch between the recipient and donor PV, graft position causing venous redundancy and kinking, prior surgery on the portal or splanchnic venous system, pre-transplant PV thrombosis (PVT) requiring thrombectomy, small diameter of the PV, use of venous conduits for reconstruction, and decreased PV flow due to the presence of porto-systemic (P-S) shunts^[2,69,71-73]. In paediatric LT and as a result of previous Kasai surgery and recurrent cholangitis, the PV becomes narrow and hypoplastic. The impaired quality of PV flow increases the risk of complications, with an incidence ranging between 3.6% and 14%^[74]. PV complications include high or low portal flow, PV stenosis (PVS) and PVT.

High portal flow: As previously described, IO and early PO benign elevation of the portal flow velocities and flow volume are expected after LT; however, there are no cut-off DU parameters that would define the need for surgical intervention. Therefore, it is important to correlate the Doppler measurements with the graft volume and the portal venous pressure (PVP) in such situation. When a high portal flow is accompanied by a suboptimal graft recipient weight ratio, hyperperfusion injury and small for size syndrome may result where the portal flow should be modulated in

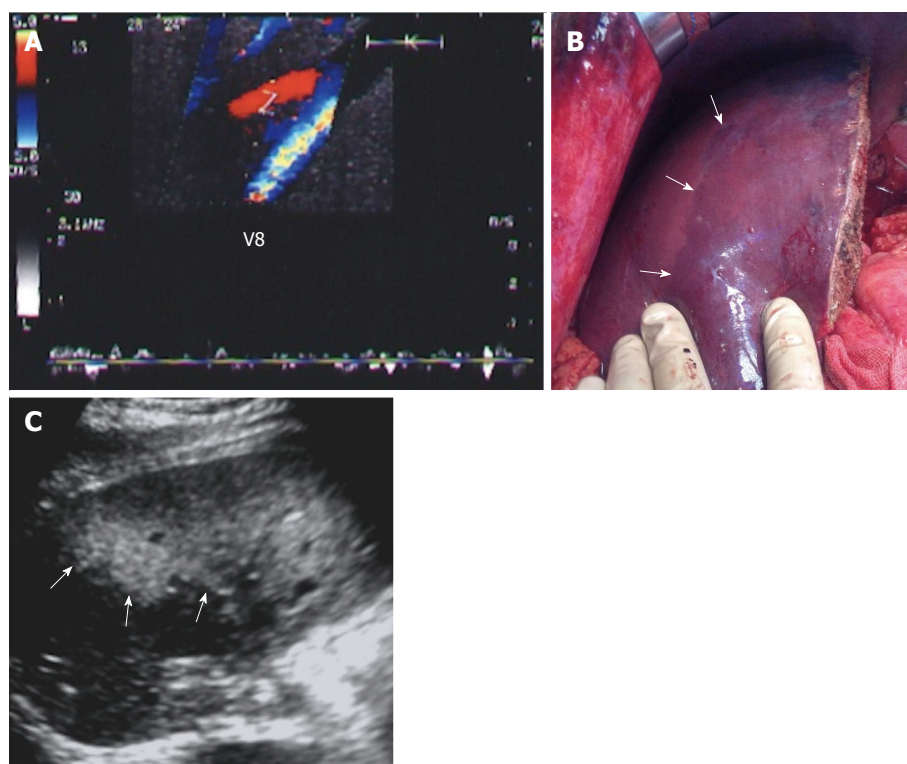


Figure 16 Graft congestion secondary to segment VIII vein ligation. A: Intra-operative color Doppler image showing stagnant (to-and fro flow) within a ligated V8 (normal intraoperative finding); B: Photography showing geographical areas of discoloration (congestion) along the anterior segments of the graft (arrows); C: Postoperative US image of the same patient 2 d post-operative demonstrating large, echogenic, geographical areas of congestion along the anterior segments of the graft (arrows). Associated graft dysfunction was present. V8: Segment VIII vein.

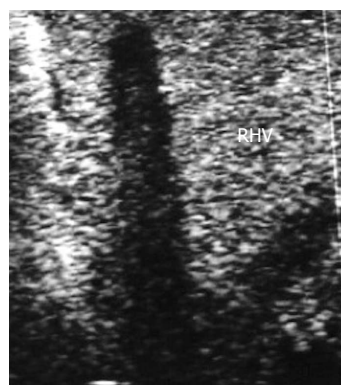


Figure 17 Intra-operative right hepatic vein thrombosis. B-mode, IOUS image showing echogenic shadows and total thrombosis of the RHV. Surgical correction was futile. RHV: Right hepatic vein.

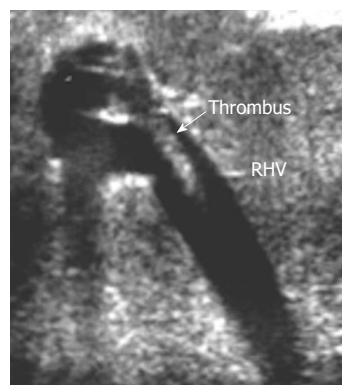


Figure 18 Intra-operative right hepatic vein partial thrombosis. B-mode, IOUS image showing small adherent thrombus near the RHV anastomosis without impeding the blood flow, and was managed conservatively. RHV: Right hepatic vein.

such cases^[75,76]. In addition, a PVP > 20 mmHg in the early period showed close associations with morbidity and poor graft function^[77]. Splenic artery ligation and portocaval shunts have been described to reduce the portal flow^[35,75].

Low portal flow: Reduced portal flow after graft perfusion may predispose to graft dysfunction and PVT. Low portal flow may occur due to the presence of P-S shunts that could be large enough to cause steal phenomena or the creation of porto-caval shunts

for portal flow diversion in small for size grafts; the condition may also occur secondary to HV outflow obstruction. A PV flow velocity of less than 10 cm/s has been considered unacceptable and requires intervention. Repositioning the graft to relieve graft torsion and kinking of the PV may allow adequate blood flow to the graft, as can collateral shunt ligation, anastomosis revision, inferior mesenteric vein cannulation and endovascular stent placement^[74,78,79]. Doppler US can provide accurate guidance when

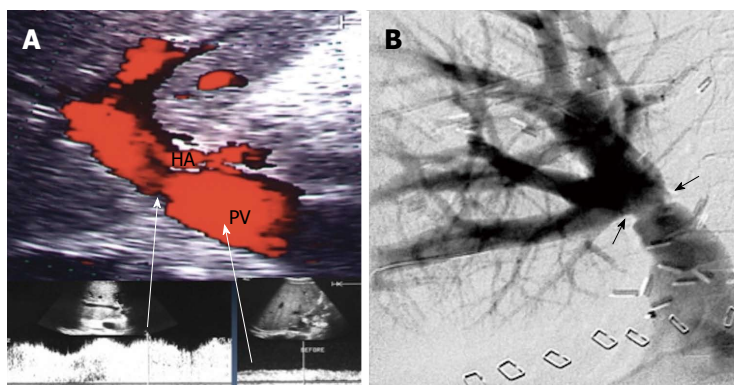


Figure 19 Portal vein pseudo-stenosis in a clinically silent patient. A: Post-operative color Doppler image, 2 mo post living donor liver transplantation demonstrating anastomotic stenosis 50%-60%, with elevation of the anastomotic velocity (4-fold) compared to the pre-anastomotic velocity; B: Percutaneous trans-hepatic digital subtraction portography image, showing anastomotic stricture (arrows) however, the pressure gradient across the anastomosis was 3 mmHg and angioplasty and stent placement were not performed. PV: Portal vein; HA: Hepatic artery.

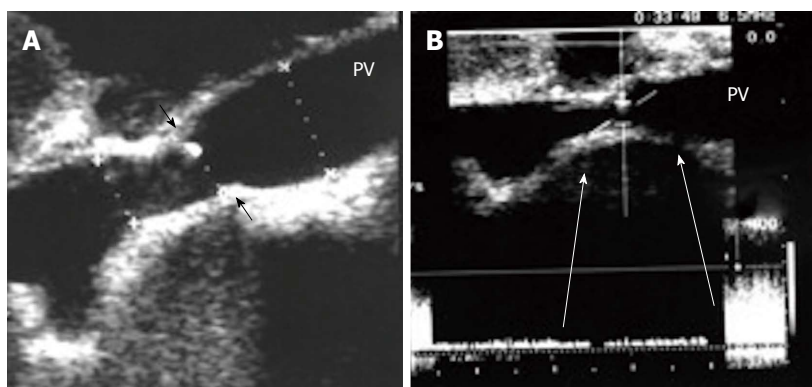


Figure 20 Intra-operative portal vein stenosis. A: B-mode US image showing PV anastomotic stricture; the suture line is seen encroaching on the lumen (arrows) with high level echoes seen at the site of anastomosis, representing turbulence of flow; B: Pulsed Doppler image demonstrating anastomotic elevation of the PV velocity (6-fold) compared to the pre-anastomotic velocity. The pressure gradient across the anastomosis was 8 mmHg, indicating the need for surgical anastomotic revision. PV: Portal vein.

monitoring portal flow during these manipulations^[79]. Post-operatively, heparinized saline infused *via* the IMV catheter to increase the PV flow and to lower the risk of thrombosis have been described^[80].

Portal vein stenosis: With advancements in surgical innovation, PVS in LDLT is relatively uncommon, with an incidence of < 3%^[66]. IO and early PO PVS usually result from technical errors or early PO anastomotic edema, whereas late PO PVS is usually secondary to fibrosis or intimal hyperplasia^[81]. The majority of patients with PVS are asymptomatic and the diagnosis of PO stenosis is an incidental finding detected on routine DU screening and can be referred to as pseudo-stenosis (Figure 19). PVS usually develops slowly after transplantation and is suspected on the basis of the presence of manifestations of portal hypertension, such as gastrointestinal varices, ascites and splenomegaly^[78,82-86]. Liver function tests and graft failure are not consistently reliable signs for PVS diagnosis^[66].

It is necessary to differentiate between PVS

and PV size mismatch by DU. PV calibre differences appear on b-mode US as tapering of the anastomosis without encroachment of the suture line on the lumen. By pulsed Doppler, elevations of the anastomotic velocities (usually < 3-fold) compared to the pre-anastomotic velocities are apparent (Figure 11). In PVS, encroachment of the suture line on the lumen can cause focal color aliasing with > 3-fold elevation of the anastomotic velocities by Color Doppler flow (Figures 20, 21 and 22). Haemodynamically significant stenosis may produce post-stenotic aneurysmal dilation as well^[78] (Figure 23). A PV stenotic ratio has been proposed to define the degree of stenosis using the formula: PV stenotic ratio (%) = pre-stenotic calibre-anastomotic calibre/pre-stenotic calibre. Anastomotic calibre < 5 mm and stenotic ratio > 50% have been defined as the critical points for PVS cases that require interventional management for good long-term graft survival^[87]. In equivocal cases, MDCT can confirm the morphological degree of the stricture and other signs of portal hypertension but will not provide quantitative information about the degree of stricture. A PVP

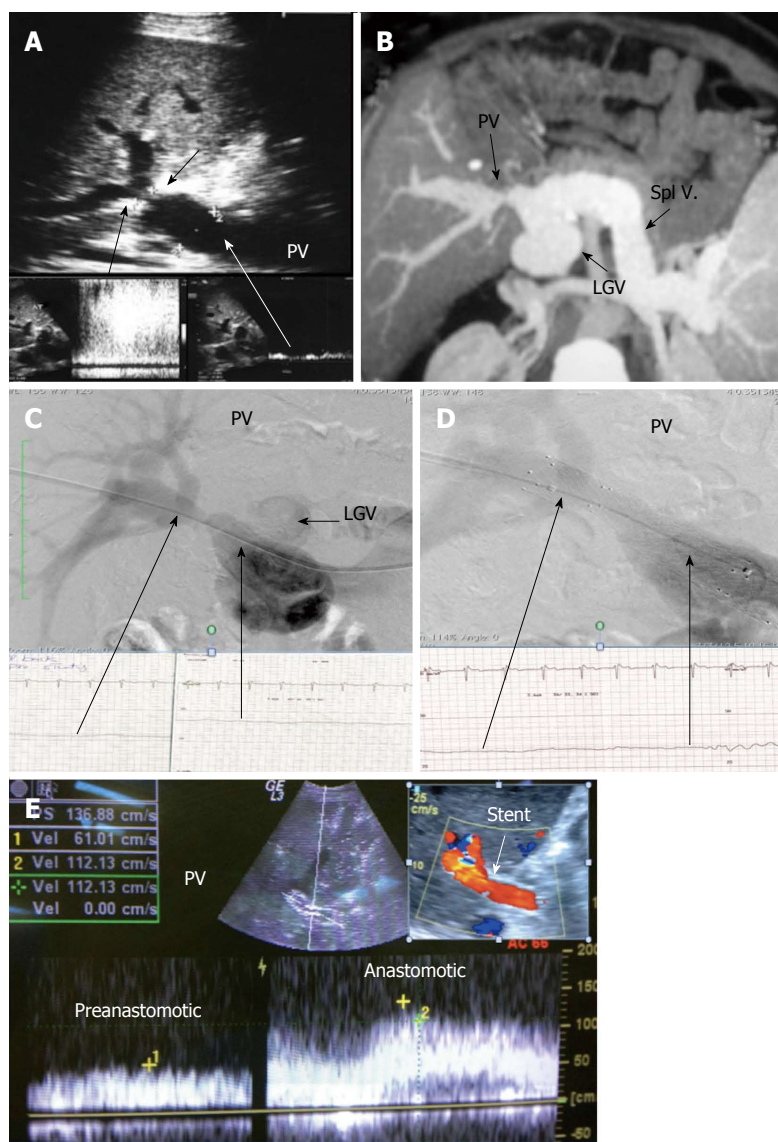


Figure 21 Post-operative developmental portal vein stenosis in a patient with attack of variceal bleeding, 1-year post-living donor liver transplantation. A: B-mode and pulsed Doppler image showing tight PV anastomotic stricture (arrows) and anastomotic jet (> 10 -fold) compared to the pre-anastomotic velocity; B: MSCT angiography image demonstrating tight PV anastomotic stricture with aneurysmal dilatation of the left gastric vein; C: Percutaneous trans-hepatic portography demonstrating tight PV stricture and dilated left gastric vein, stealing the portal flow. The pressure gradient was 10 mmHg; D: Control portography after balloon dilatation and stent placement demonstrating wide anastomosis, normalization of the pressure gradient, improvement of the flow steal and disappearance of the left gastric vein filling; E: Color Doppler image after PV stent placement confirming improved portal flow with increased pre-anastomotic velocity and mild elevation of the anastomotic velocity. PV: Portal vein; LGV: Left gastric vein.

gradient across the anastomosis can be performed in the recipient surgery or during the PO interventional angioplasty procedures, pressure gradients > 3 -5 mmHg are considered significant and necessitate intervention^[81,88] (Figure 21).

In patients with clinical manifestations and radiological confirmation of significant stenosis, therapeutic intervention is mandatory. Interventional angioplasty and stent placement have become widely recognized as the first choice for treatment^[80,81,89] (Figures 21 and 22).

Portal vein thrombosis: PVT is a severe complication that may occur during either the recipient surgery or

PO. Early identification by DU allows early intervention to avoid prolonged warm ischaemia, which may result in graft failure. The incidence of PVT in OLT ranges from 0.3%-2.6%^[1,4,69], and a higher incidence up to 4% has been reported in LDLT due to technical difficulties in PV reconstructions, mainly related to shorter vessel pedicles and limited vessel grafts. The condition occurs more frequently in the early PO period, defined as within 3 mo after transplantation^[71]. The clinical presentation depends on the timing of thrombosis. If the condition occurs early, severe acute liver insufficiency or graft failure predominates; if the condition occurs late, portocaval collateral circulation usually exists and the patient may present with

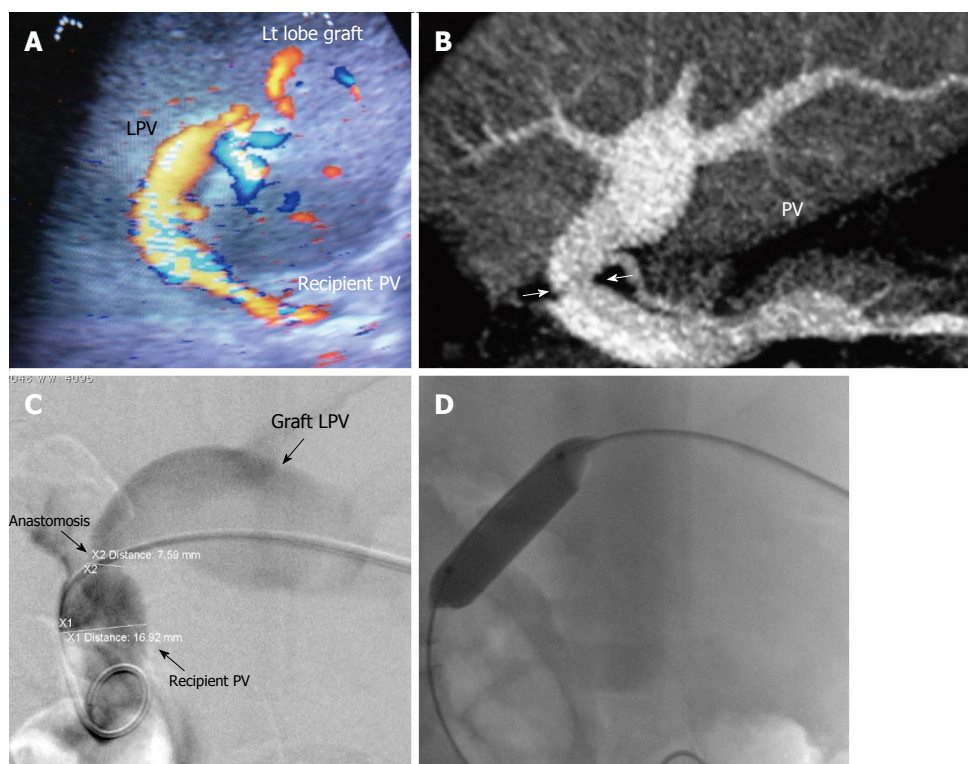


Figure 22 Post-operative developmental portal vein stenosis in a 16-year-old boy with progressive splenomegaly and pancytopenia, 1-year post-left lobe living donor liver transplantation. A: Color US image showing PV anastomotic stricture and post-stenotic dilatation. The anastomotic velocity elevation was borderline (3–4-fold) compared to the pre-anastomotic velocity (not shown); B: Multi-slice CT angiography, reconstructed image, demonstrating moderate PV anastomotic stricture with post stenotic dilatation of the graft PV; C: Percutaneous trans-hepatic digital subtraction portography image, demonstrating moderate PV anastomotic stricture (> 50 %) with post-stenotic dilatation. The pressure gradient was 7 mmHg; D: X-ray image during balloon dilatation of the stricture. The pressure gradient was normalized and stent placement was not performed. PV: Portal vein; LPV: Left portal vein.

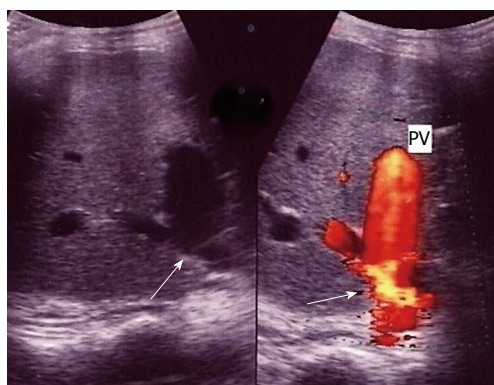


Figure 23 Post-operative developmental portal vein stenosis 2-years post-living donor liver transplantation. B-mode and color US image demonstrating tight PV anastomotic stricture with color aliasing (arrows) and aneurysmal dilatation of the PV anterior segmental branch. PV: Portal vein.

manifestations of portal hypertension^[69,73,90].

IO total PV thrombosis is rare whereas, detection of residual adherent thrombi in the recipient PV after thrombectomy is common for pre-existing PVT. Old thrombi appear as echogenic filling defects that adhere to the wall (Figure 24). Thrombi that are small in size and do not cause flow obstruction may be conserved; large thrombi that impede the flow necessitate further thrombectomy. On b-mode US, acute PVT appears

as a hypo-echoic or slightly echogenic shadow within the PV with absent flow by color Doppler (Figures 25 and 26). Proper color Doppler evaluation of portal flow is crucial so that a slow portal flow is not mistaken as thrombosis^[7,78]. Secondary hepatic infarction may be observed on US as an irregular, wedge-shaped low attenuation areas of heterogeneous echo pattern and devoid of vascular activity that are mainly in the periphery of the liver. Those areas appear as perfusion defects on CT examination (Figure 26B). In late onset or gradual PVT, portal cavernoma may be observed at the graft hilum with attenuated or non-visualized main graft PV (Figure 27).

In early PVT, within the first 3 d, surgical revision of the anastomosis is mandatory, together with systemic anticoagulation^[2]. In more delayed cases, non-surgical treatment, such as percutaneous thrombolysis and stent placement, has shown good results and acceptable safety^[2,91–94].

Hepatic artery complications

Arterial complications are the most common VCs after LT that can lead to ischaemia and graft loss with high mortality and morbidity rates^[3,95,96]. Anatomical variations of the HA, diameter and length, kinking, quality of the recipient artery and mismatch between donor and recipient arteries should be carefully

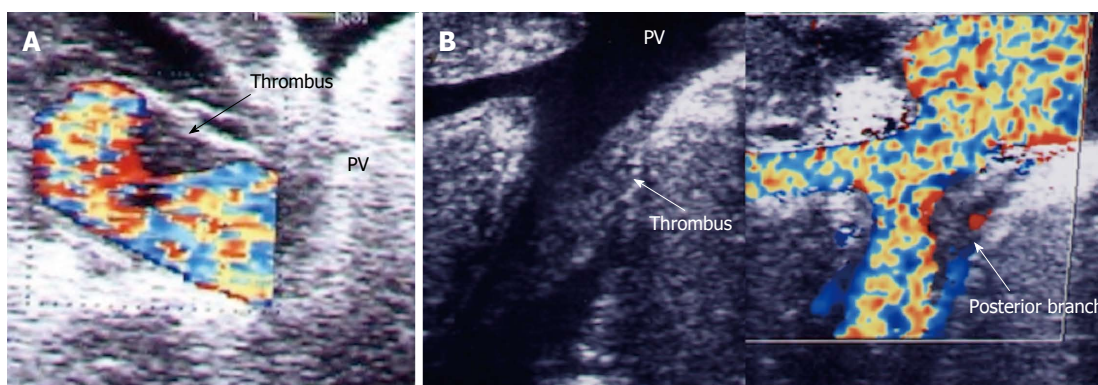


Figure 24 Intra-operative portal vein thrombosis. A: Intra-operative color US image showing a small adherent thrombus to the PV wall, persisting after thrombectomy for old subtotal PV thrombosis. There was good portal flow and was managed conservatively; B: Intra-operative B-mode and color US image of another patient showing acute thrombus adherent to the PV posterior segmental branch inside the graft. Thrombectomy failed to extract the thrombus and was managed conservatively. PV: Portal vein.

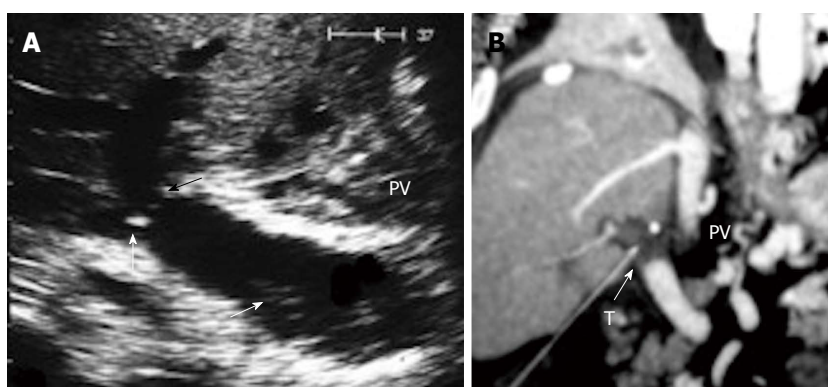


Figure 25 Post-operative portal vein thrombosis, 1 wk post-living donor liver transplantation. A: B-mode US image showing faintly echogenic thrombus in the recipient PV (arrow) and PV anastomotic stricture (arrow heads); B: Multi-slice CT angiography, coronal reconstructed image after 2 h, demonstrating total PV thrombosis (arrow). The patient underwent thrombectomy and anastomotic revision. PV: Portal vein; T: Thrombus.

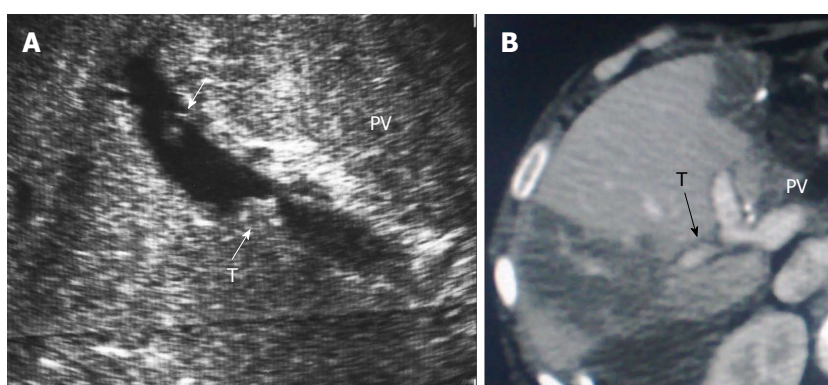


Figure 26 Post-operative portal vein segmental branch thrombosis and subsequent graft infarction, 2 wk post-living donor liver transplantation. A: B-mode US image showing multiple acute thrombi in the posterior segmental branch of the PV; B: Triphasic portal venous CT contrast study, demonstrating attenuated posterior branch of the PV with filling thrombus defect (arrow). Segmental portal infarction appears as wedge-shaped area of hypo-perfusion. PV: Portal vein; T: Thrombus.

considered during DU interpretation and can be managed accordingly^[78,97]. HA complications include vasospasm, HA thrombosis (HAT), HA stenosis (HAS), HA dissection, arterial steal phenomena and pseudoaneurysm formation.

Hepatic artery vasospasm: Vasospasm is a state

of arterial constriction; the affected segment or entire vessel becomes rigid and its lumen narrows or even becomes occluded^[98,99]. It is a common complication during the recipient surgery secondary to excessive manipulations and suturing. The results of animal and clinical studies suggested that the plasma norepinephrine level is elevated because of surgical

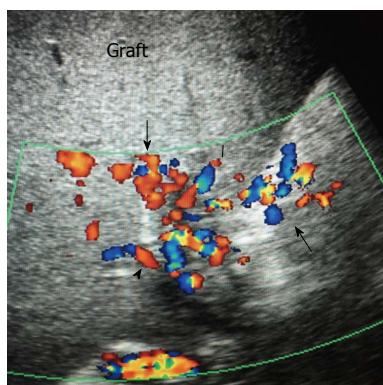


Figure 27 Chronic post-operative portal vein thrombosis, 2-years post-living donor liver transplantation. Color US image showing numerous dilated collateral venous channels at the graft hilum (arrows) and non-visualized main graft portal vein.

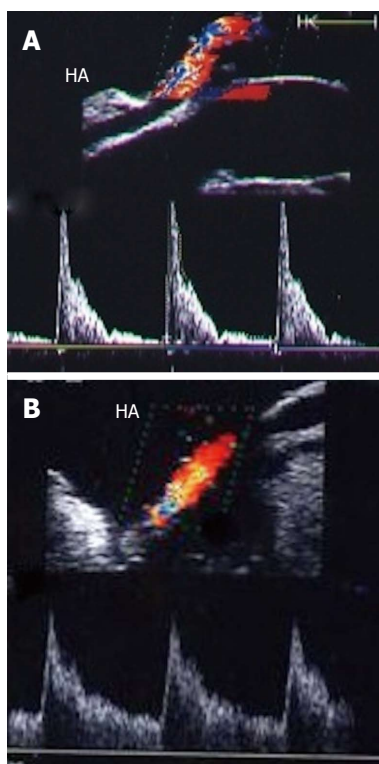


Figure 28 Intra-operative hepatic artery vasospasm. A: Intra-operative Doppler US image showing high resistance flow in the HA with low diastolic flow and normal systolic upstroke; B: Doppler US image of the HA after intra-operative papaverine installation showing relief of spasm, regaining the normal biphasic Doppler waveform, and a continuous diastolic flow throughout the cardiac cycle. HA: Hepatic artery.

stress, occlusion of hepatic inflow and removal of the donor liver, which might have an appreciable effect on donor arterial spasm^[100-102]. The potential impact of vasospasm in transplants is still undetermined, it is possible that vasospasm may cause reduction of HA flow and may induce the formation of thrombosis^[99,103]. Richards *et al*^[104] pointed out that vasospasm in re-vascularized tissue could impair tissue perfusion even though the microsurgical anastomoses remain patent.

There is lack of standard diagnostic criteria of HA vasospasm by Doppler US; it may appear as a short attenuated segment of the artery without focal anastomotic narrowing and usually results in increased RI, sometimes with absent or reversed diastolic flow, and can be differentiated from other causes of increased RI, previously described, that it responds to IO local application or PO administration of vasodilators (Figure 28). The increased RI can be explained according to Poiseuille's equation, $R = 8\lambda\eta/\pi r^4$, the resistance (R) depends on the viscous properties of the blood (η) and on the dimensions of the vessels. Whenever the hepatic artery is contracted, its diameter will correspondingly reduce, and, as a result, the resistance of the hepatic arterial flow will increase^[103,105]. The diagnosis of HA vasospasm can be also supported by the follow-up PO DU results, and can be used to monitor the dynamic changes of the involved arteries before and after vasodilatation^[103] (Figure 29).

Hepatic artery thrombosis: HAT is the most frequent VC following LT occurring with an incidence of 1%-12%, and is a graft-threatening condition that necessitates rapid revascularization to avoid graft loss^[1,65,95,106]. Potential mechanical risk factors include vasospasm, dissection, kinks, significant mismatch, anastomotic stricture, arterial steal, bench reconstruction, arterial conduit use and multiple anastomoses^[106]. Nonsurgical risk factors include hyper-coagulable disorders, cytomegalovirus, ABO incompatibility, arterial reperfusion time, prolonged operation times and acute rejection^[107,108]. Early HAT within the first few weeks PO usually presents with fulminant graft failure and sepsis whereas delayed thrombosis occurring after the first month has a more insidious clinical course with delayed biliary complications^[106].

Acute HAT can be diagnosed by b-mode US as a hypo-echoic or an-echoic shadow inside the artery and complete absence of color flow and arterial wave by pulsed Doppler (Figures 30 and 31). However, some earlier haemodynamic changes may occur and can anticipate total occlusion; these changes are referred to as signs of impending thrombosis^[41]. Some authors have observed that absent or reversed diastolic flow appeared just before the HA flow vanished in patients with HAT^[41,42,103] (Figure 32), indicating that under certain circumstances, HAT may share a common DUS appearance with vasospasm^[103]. However, other authors found this sign to be poorly associated with HAT because the incidence of HAT was low among patients who had this highly resistant arterial flow^[109]. In our experience, dampening of the PSV can differentiate between impending thrombosis and vasospasm; in vasospasm, the PSV is usually high, with good systolic upstroke, whereas in impending thrombosis, the systolic upstroke is usually damped and irregular (Figure 32). Chen *et al*^[103] differentiated

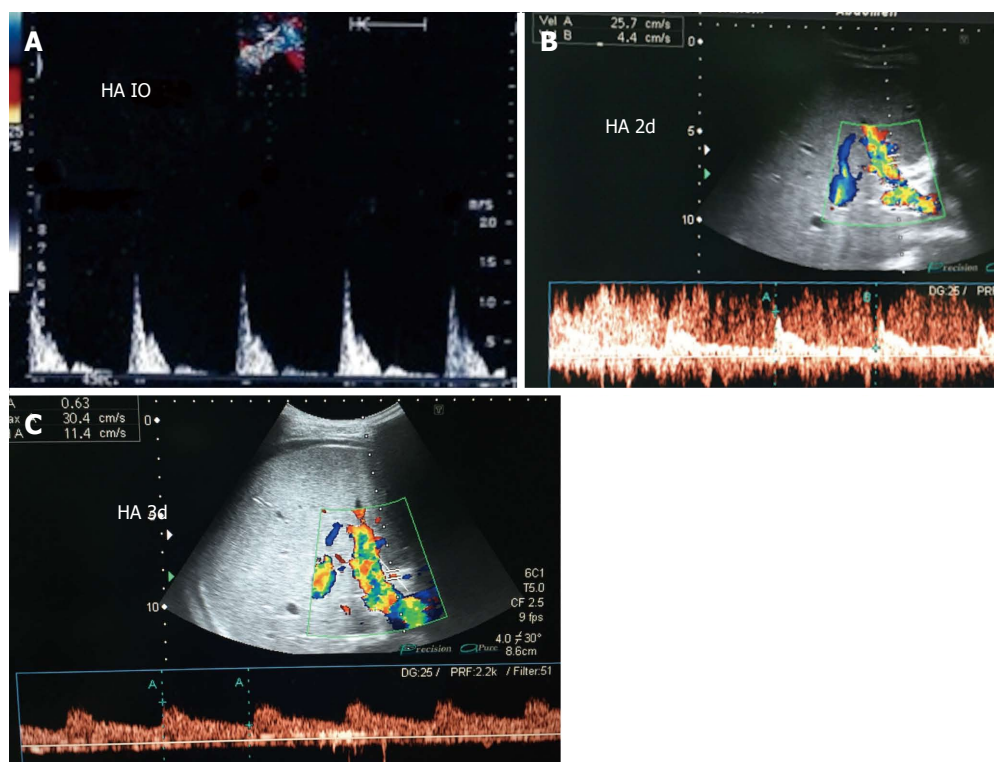


Figure 29 Intra- and post-operative hepatic artery vasospasm. A: Intraoperative Doppler US image showing high resistance of the HA with damped diastolic flow and normal systolic upstroke; B and C: Doppler US images of the HA, 2 d and 3 d post-operative after systemic administration of vasodilators, showing improvement of the diastolic flow and correction of the normal biphasic Doppler waveform. HA: Hepatic artery; IO: Intra-operative.

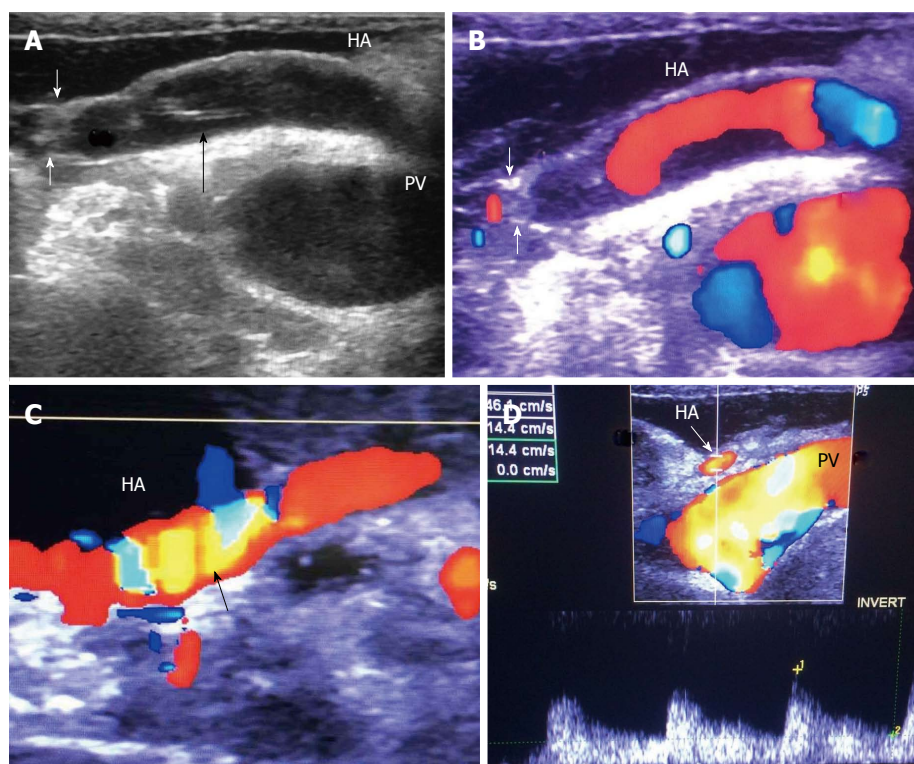


Figure 30 Intra-operative hepatic artery dissection and thrombosis. A: IOUS B-mode image, showing HA anastomotic stricture (White arrows) and a dissecting flap at the recipient side of the artery (black arrow); B: Color image showing abrupt occlusion of the HA proximal to the anastomosis and acute thrombosis at the site of anastomosis; C: Color image after revision of the anastomosis, showing patent artery with color aliasing at the region of the anastomosis (arrow); D: Color Doppler image, showing normal biphasic arterial flow distal to the anastomosis. HA: Hepatic artery; PV: Portal vein.

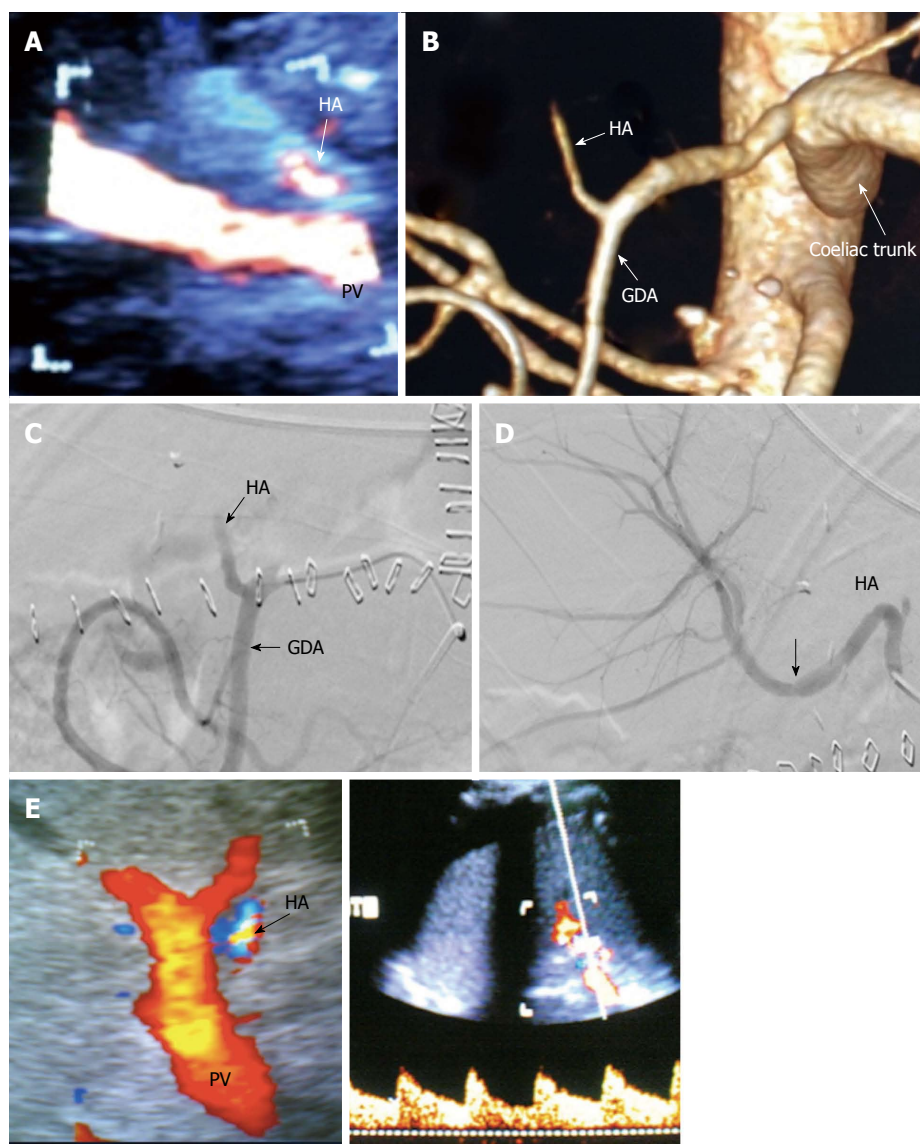


Figure 31 Post-operative hepatic artery thrombosis 4 d post-living donor liver transplantation in a patient with shooting liver enzymes. A: Color US image, showing abrupt occlusion of the HA at the graft hilum (arrow) and absence of intra-hepatic flow; B: MDCT angiography, volume rendering image, showing complete occlusion of the HA proximal to the anastomosis and non-filling of the intra-hepatic branches (arrow); C: Digital subtraction angiography image, confirming complete thrombosis of the HA proper (arrow); D: Control angiography after thrombolysis with tissue plasminogen activator, showing resolution of the thrombus and filling of the intrahepatic branches. There was mild size mismatch noted at the anastomotic site (arrow) with no underlying stricture; E: Color Doppler image performed during the procedure after HA recanalization, showing normal biphasic flow inside the graft confirming absence of significant stenosis, therefore stent placement was not necessary. HA: Hepatic artery; GDA: Gastroduodenal artery.

between impending thrombosis and vasospasm by the absence of diastolic flow in the main HA and in its parenchymal branches in vasospasm, whereas in impending thrombosis, the absent diastolic flow occurs around the anastomotic site only. HAT requires re-transplantation in a majority of cases, whereas surgical revision and endovascular intervention are the primary options if DDLT is not available^[2,3,95,110].

Hepatic artery dissection: Arterial dissection involves the separation of the intimal lining of an artery from the media and, less frequently, the separation of the media from the adventitia. Dissection is usually accompanied by haemorrhage into the arterial wall,

which creates a blind pouch or a parallel sub-intimal second channel or false lumen^[111]. HA dissection is a predisposing factor to arterial stenosis and occlusion and usually occurs secondary to surgical trauma and clamp injury. It can occur in either the recipient or the donor side of the artery.

IO dissection of the recipient artery is not uncommon, especially in hypertrophied, diseased arteries in elderly patients. The dissection is usually self-limiting because the direction of blood flow seals the tear and can be managed either by anastomotic revision or using another recipient artery^[112]. However, dissection of the graft artery is more dramatic as the direction of flow increases the dissection and can extend to the

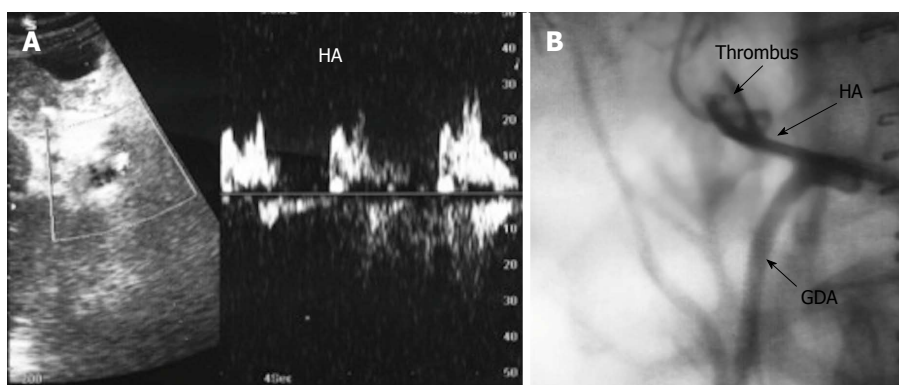


Figure 32 Post-operative hepatic artery impending thrombosis in a patient with stable liver enzymes, 3 d post-living donor liver transplantation. A: Doppler US image, showing abnormal arterial waveform with damped irregular systole, absent and reversed diastolic flow (suspicious of impending thrombosis); B: Arterial angiography performed after 2 h showing acute thrombosis and dilatation of the recipient HA proper. Intra-arterial thrombolysis was performed successfully. HA: Hepatic artery.

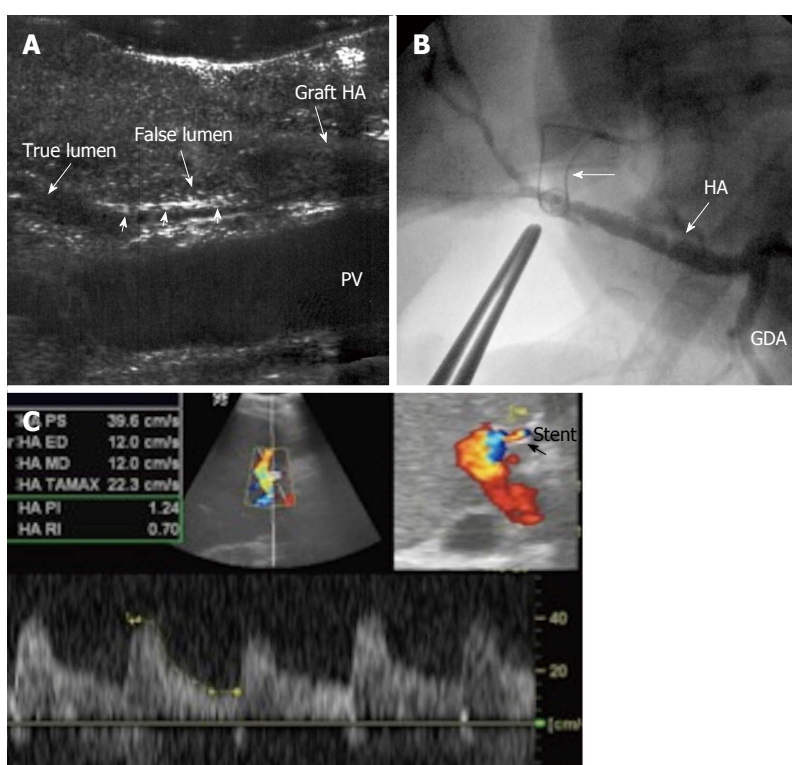


Figure 33 Intra-operative dissection of the graft hepatic artery. A: IOUS B-mode image, showing dissecting intimal flap within the graft artery (arrow heads), the false lumen is seen echogenic and filled with blood and the flap totally occludes the true lumen. Surgical correction failed to repair the dissection; B: Hepatic arteriography image after successful hybrid endovascular stent placement, showing patent HA and good intrahepatic flow. A metallic marker was applied to mark the anastomotic site before stent placement (arrow); C: Doppler US image 4 mo later, confirmed patency of the stent with normal arterial waveform. HA: Hepatic artery.

intrahepatic branches, eventually occluding the artery and requiring repair (Figure 33).

The most common US finding of HA dissection is a double lumen that is separated by an echogenic intimal flap or two parallel intraluminal flaps in cases of circumferential intimal dissection (Figure 34A). An intramural haematoma can also be demonstrated as an eccentric echogenicity that surrounds a relatively narrowed arterial lumen. Color Doppler may demonstrate the true and false lumina. Surgical anastomotic revision of a dissected HA is mandatory in the absence of arterial flow, in the presence of low flow

velocity or if the flow is seen in both the true and false lumina with double systolic peaks, signifying ongoing dissection (Figure 34B).

When DU shows a single stream from the main lumen with a sealed or thrombosed false lumen, the anastomosis may not be revised^[95] (Figure 35).

Arterial steal phenomena: Arterial steal syndromes have been described as a cause of arterial hypoperfusion after LT. These syndromes are characterized by low arterial flow towards the graft, caused by shift of flow into either the splenic artery or the

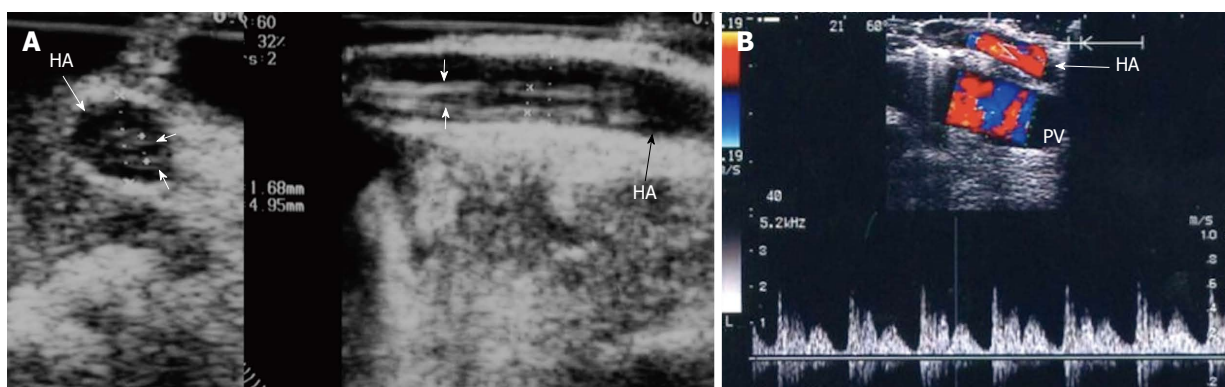


Figure 34 Intra-operative dissection of the recipient hepatic artery. A:IOUS B-mode image in axial and longitudinal scans, showing circumferential dissection of the recipient HA with 2 parallel echogenic intimal flaps seen within; B: Color Doppler image, showing a characteristic double-hump early presystolic pulse waveform, signifying ongoing dissection, that necessitates revision of the anastomosis. HA: Hepatic artery.

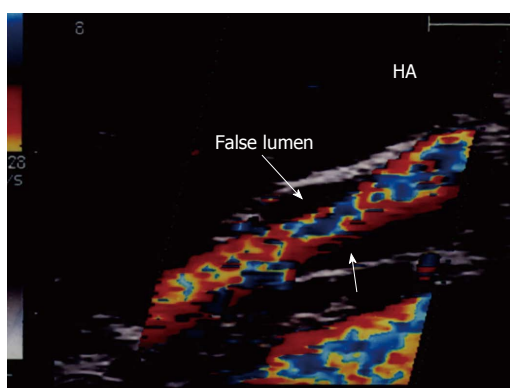


Figure 35 Intra-operative dissection of the recipient hepatic artery. IOUS color image showing recipient HA dissection (proximal to the anastomosis). The dissection was self-limiting; a single stream flow in the true lumen and thrombosed false lumen (sealed, arrows). Therefore, revision of the anastomosis was not necessary. HA: Hepatic artery.

gastro-duodenal artery, and are usually diagnosed angiographically^[95,113]. The PO DU findings describing these phenomena are nonspecific, such as loss of HA flow signal, decreased HA PSV, elevated or reduced RI, and total absence of diastolic flow^[114-116]. Data regarding IO arterial steal are lacking, though in our experience, a low arterial flow coming from the HA proper may be due to coeliac artery stenosis, arcuate ligament syndrome or arterial steal. Experimental clamping of the gastroduodenal artery and the splenic artery may be performed to diagnose arterial steal (Figure 36).

Hepatic artery stenosis: HAS can cause complications similar to HAT, but the condition is less grave with a more insidious course, occurring in 2% to 13% of transplants^[2,50,117]. IO recognition of HAS is extremely important, especially in significant cases, because by itself, it causes graft ischaemia. Untreated stenosis can progress to the even more devastating HAT, early graft failure or acute bile duct necrosis and biliary sepsis^[118,119]. Post-operatively, patients' with

mild HAS may be asymptomatic or may present with abnormal liver enzymes or biliary strictures.

On DU, HAS can be diagnosed by direct visualization of the anastomotic narrowing by b-mode, anastomotic velocity jet with turbulence and aliasing and distal dampening of the flow (Figures 37 and 38). The PSV at the stenotic segment is usually elevated to greater than 200 cm/s^[57,120,121]. Vascular kinks due to vessel redundancy can lead to marked elevation of the PSV, where correction of the angle of interrogation can help differentiate from true stenosis. Anastomotic edema may also cause temporarily increased HA velocity mimicking HAS in the immediate PO scans^[40].

Secondary changes seen downstream of the stenosis are very useful in establishing the diagnosis, they are very reliable if there is technical difficulty in direct visualization of the anastomosis due to kinks or small arteries. Distal to the stenosis, the resistance of the arterial tree decreases, resulting in increased diastolic flow and a corresponding decrease in RI to less than 0.55. The rapid hepatic arterial systolic upstroke gets delayed with an increased systolic acceleration time ≥ 0.08 second (tardus parvas waveform), and the sensitivity and specificity of the secondary changes range from 73%-83% and 60%-73%, respectively^[121-124]. In the PO period, the tardus parvas waveform has a false-positive diagnosis due to conditions other than HAS, including severe aorto-coeliac atherosclerotic disease, arterio-venous or arterial-biliary fistula formation, and hepatic venous or portal venous thrombosis^[125,126].

In our practice we do not rely on the absolute value of the PSV at the anastomosis as it is influenced by many factors, including the systemic pressure, source of the recipient artery, size mismatch, kinks, and spasm. A high- anastomotic jet (> 3 -fold) compared with the pre-anastomotic segment with high pitched sound and an intrahepatic tardus parvas waveform are considered signs of HAS (Figure 38B). Management of HAS includes either surgical revision or percutaneous endovascular balloon dilatation and stent placement^[127,128].

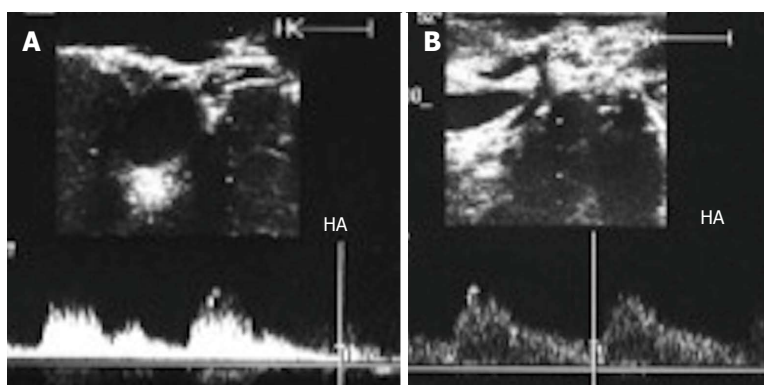


Figure 36 Gastroduodenal arterial steal. A: Intra-operative Doppler US image showing weak flow in the recipient HA (pre-anastomotic), with damped irregular systolic peak; B: Doppler US image after experimental clamping of the gastroduodenal artery, demonstrating improvement of the arterial waveform and regaining of the sharp systolic upstroke. Ligation of the gastroduodenal artery was then performed. HA: Hepatic artery.

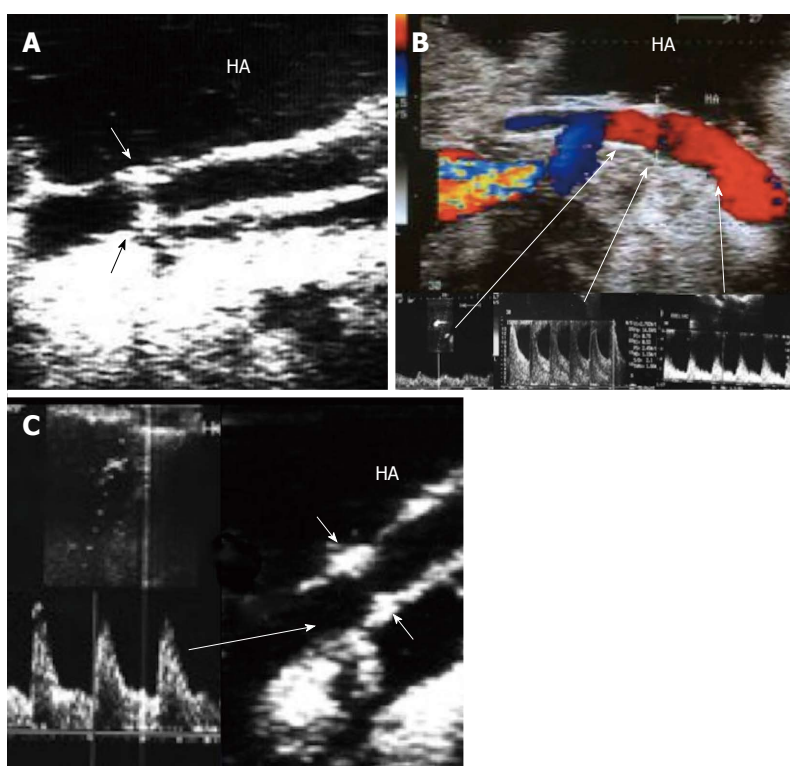


Figure 37 Intra-operative hepatic artery stenosis. A: IOUS B-mode image revealing significant stricture at the anastomotic site (arrows); B: Color Doppler image showing anastomotic jet of the systolic and diastolic arterial flow compared to the pre-stenotic segment and dampening of the distal flow (tardus parvus waveform); C: Doppler US image distal to the anastomosis after surgical revision, revealing improvement of the distal arterial flow with normal waveform (arrows). HA: Hepatic artery.

HA pseudo aneurysms and peripheral arterioportal fistula: HA pseudo aneurysm is rare complication after LT with a reported incidence of 0.27%-3%. It results in leakage of the blood outside the arterial wall into surrounding tissue. Risk factors include technical problem in the arterial anastomosis, peritoneal infections and biliary leak^[129-131]. Patients may be asymptomatic or present with abdominal pain, fever, gastrointestinal bleeding or bleeding through the abdominal drain. On sonography HAP appears as a peri-portal or cystic structure with a disorganized arterial flow pattern or a characteristic bidirectional flow^[132]. Management is either by surgical repair or

interventional radiology^[129,133].

Intra-hepatic arterio-portal fistula usually occurs following surgical biopsy or percutaneous liver biopsy or local infection and is often detected incidentally. They appear on DU as an intra-hepatic cystic structure with pulsatile portal vein branch communicating with a fistula to the arterial tree at the biopsy site^[78] (Figure 39).

CONCLUSION

Doppler ultrasonography is the primary imaging modality and the most important diagnostic tool for the evaluation of the graft vascular perfusion in LDLT, both

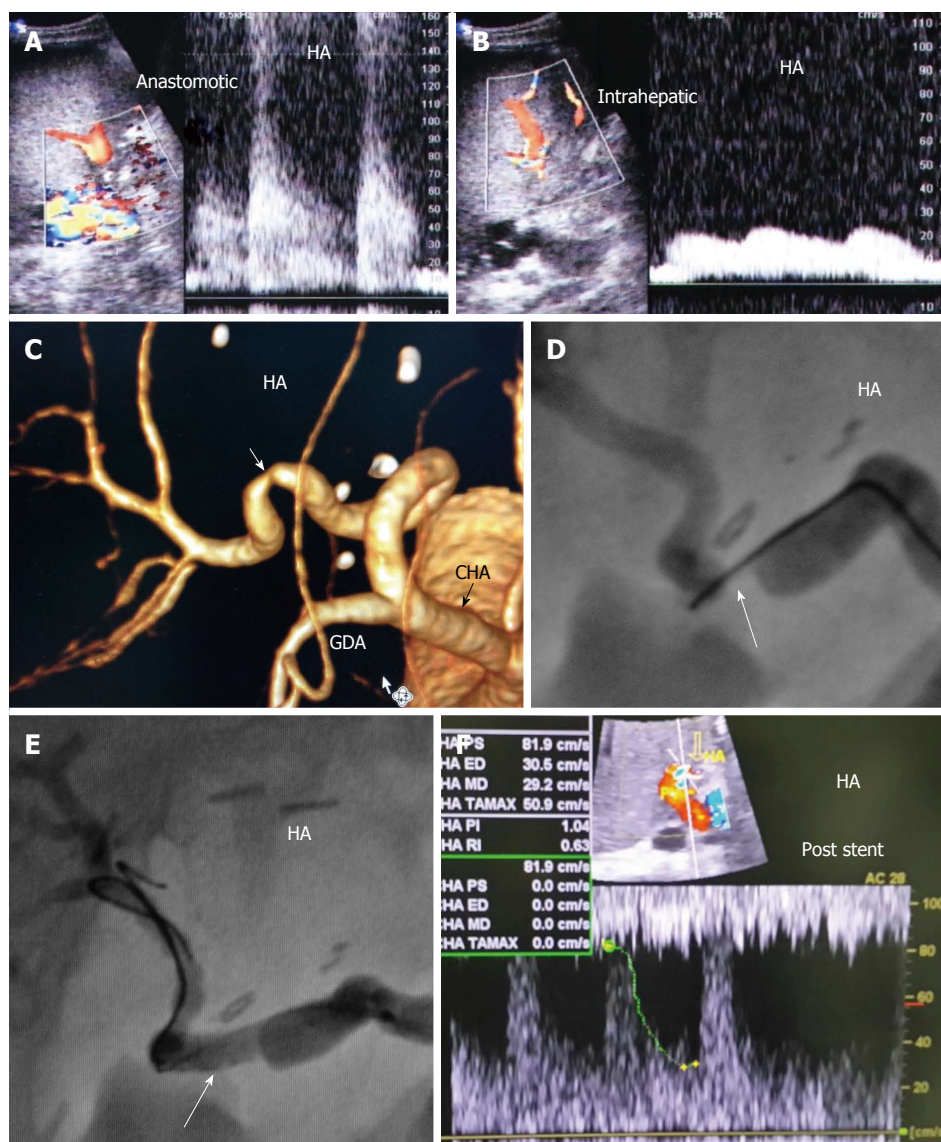


Figure 38 Post-operative hepatic artery stenosis in a patient with persistent graft dysfunction, 2 mo post-living donor liver transplantation. A: Doppler US image showing arterial velocity jet at the graft hilum; B: Doppler US image showing damped intra-hepatic arterial flow (tardus parvas waveform); C: MDCT angiography, volume rendering image, showing significant anastomotic stricture; D: Conventional angiography showing tight anastomotic stricture (arrow); E: Conventional angiography after stent placement (arrow); F: Doppler US image performed during the procedure showing normal intrahepatic arterial waveform after stent placement. HA: Hepatic artery.

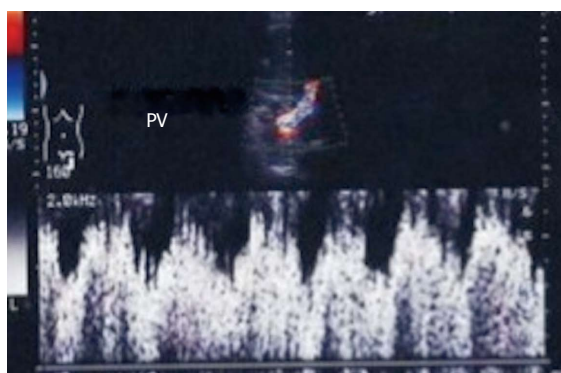


Figure 39 Post-biopsy arterio-venous fistula. Color Doppler image demonstrating a pulsatile portal vein branch in segment 6 subsequent to a post-biopsy arterio-venous fistula. PV: Portal vein.

during surgery and post-operatively. It is sensitive to blood flow dynamics and can provide the clinicians and surgeons intra-operative and post-operative potential complications that may occur. A multidisciplinary approach, early detection and thus early problem solving can make the difference between graft survival and failure.

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2016 Liver Transplantation: Global view

Advances in endoscopic management of biliary complications after living donor liver transplantation: Comprehensive review of the literature

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Abstract

Apart from noticeable improvements in surgical techniques and immunosuppressive agents, biliary complications remain the major causes of morbidity and mortality after living donor liver transplantation (LDLT). Bile leakage and stricture are the predominant complications. The reported incidence of biliary complications is 15%-40%, and these are known to occur more frequently in living donors than in deceased donors. Despite the absence of a confirmed therapeutic algorithm, many approaches have been used for treatment, including surgical, endoscopic, and percutaneous transhepatic techniques. In recent years, nonsurgical approaches have largely replaced reoperation. Among these, the endoscopic approach is currently the preferred initial treatment for patients who undergo duct-to-duct biliary reconstruction. Previously, endoscopic management was achieved most optimally through balloon dilatation and single or multiple stents placement. Recently, there have been significant developments in endoscopic devices, such as novel biliary stents, as well as advances in endoscopic technologies, including deep enteroscopy, the rendezvous technique, magnetic compression anastomosis, and direct cholangioscopy. These developments have resulted in almost all patients being managed by the endoscopic approach. Multiple recent publications suggest superior long-term results, with overall success rates ranging from 58% to 75%. This article summarizes the advances in endoscopic management of patients with biliary complications after LDLT.

Key words: Biliary complication; Endoscopic retrograde cholangiography; Endoscopic management; Living donor; Liver transplantation

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Core tip: Living donor liver transplantation (LDLT) has become an accepted therapeutic option for patients with end-stage liver disease. However, biliary complications remain the major causes of morbidity and mortality for LDLT recipients and donors. Although there are currently no reports of a clear therapeutic algorithm, many approaches have been developed to treat biliary complications, including surgical, endoscopic, and percutaneous transhepatic techniques. Endoscopic treatment is currently the preferred initial treatment for patients that have previously undergone duct-to-duct biliary reconstruction. This article discusses various aspects of endoscopic management of biliary complications that occur in LDLT.

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INTRODUCTION

Because of the shortage of deceased donor organs, living donor liver transplantation (LDLT) has emerged as a widely accepted therapeutic option for patients with end-stage liver disease. There have been noticeable improvements in the surgical techniques, graft preservation technology, and immunosuppressive therapies for this procedure. However, biliary complications remain the major cause of patient morbidity, graft loss, and mortality following LDLT^[1-6]. Although the overall incidence of biliary complications in LDLT recipients has gradually declined leading to a considerable drop since 2008^[6], many investigators have reported recently that approximately 15%-40% of adult recipients will develop biliary complications after LDLT, with considerable variation among transplant centers^[5,7-13].

Spectrum of biliary complications in LDLT

Biliary complications from an LDLT procedure include biliary stricture, bile leakage, biloma, bile duct obstruction (with stones, sludge, or casts), sphincter of Oddi dysfunction, hemobilia, and mucocele^[14-16]. Among these, bile leakage and anastomotic stricture are the predominant complications^[7,17,18]. Patients often develop more than one complication^[16,19].

Biliary strictures have been reported to develop in 18%-32% of LDLT patients regardless of the graft type^[2,8,9,20-26]. Although a stricture can present at any time after transplantation, the median time interval between LDLT and the onset of biliary stricture was 5.9 mo^[27]. Approximately 70%-87% of biliary strictures occur within one year of LDLT^[28]. Biliary strictures are

classified according to their location into anastomotic or non-anastomotic^[29]. Anastomotic stricture is single and is caused by localized fibrosis due to the operative technique, postoperative bile leakage, or peribiliary ischemia^[14]. Posttransplant biliary stricture occurs primarily at the anastomotic site, and it is the most common surgical complication of LDLT^[12,19,21,30]. In contrast, non-anastomotic strictures are usually multiple and more diffuse, involving the hilum and intrahepatic bile duct^[14,19,31]. They are thought to be the result of ischemic-, immunologic-, and bile salt-induced cytotoxic injuries^[14,32,33]. With the benefit of the short ischemic time and the donor being immunogenetically healthy, there are very few reports of non-anastomotic strictures after LDLT^[33].

Bile leakage can originate from the anastomotic site, remnant cystic duct stump, T-tube tract, cut surface of the graft, or a damaged accessory bile duct^[14,19,31]. Similar to strictures, anastomotic leakage often results from vascular insufficiency or ischemic injury^[19]. The incidence of bile leakage after LDLT ranges between 5% and 18%^[9,21,22,25,28]. In one series, bile leakage comprised 65% of the LDLT patients with posttransplant biliary complications^[13]. Bile leakage is a complication that predominates in the early period after LDLT, and in 70% of cases it is found within the first month after LDLT^[30]. The median time interval between LDLT and bile leak was 0.7 mo^[27]. Bile leakage can be classified as early or late. Early leakage is usually detected at the anastomotic site and is often related to technical problems. Late leakage, although an infrequent event, is typically associated with the removal of the T-tube^[14,19,31] and may be accompanied by severe stricture due to a chronic inflammatory reaction^[34]. As the bile leakage grows, extravasation of bile can result in a biloma, as a form of intrahepatic bile lake, extrahepatic bile collection, and abscess. Most bilomas encountered after LDLT are in the perihepatic space^[19]. It is usually associated with a disconnected or strictured bile duct^[14,31].

Bile duct stones, sludge, and casts, together called bile duct filling defects, occur in approximately 5% of patients after LDLT^[12,29,31,33]. The majority of such defects are caused by stones^[19]. Bile duct stones appear a median of 19 mo after LDLT^[27], and casts present within the first year after transplantation, usually within 16 wk^[35]. Theoretically, any condition that can obstruct bile flow can predispose to stones, sludge, and casts^[14,33]. These filling defects are seen in strong association with ischemic events and are often accompanied by other biliary complications, most commonly biliary stricture^[19,35-37]. In general, patients with persistent biliary stricture due to an ischemic etiology often manifest with recurrent intrahepatic biliary stones and sludge. Stones and sludge repeatedly accumulate proximal to the stricture, which leads to the formation of casts and a high incidence of cholangitis^[38].

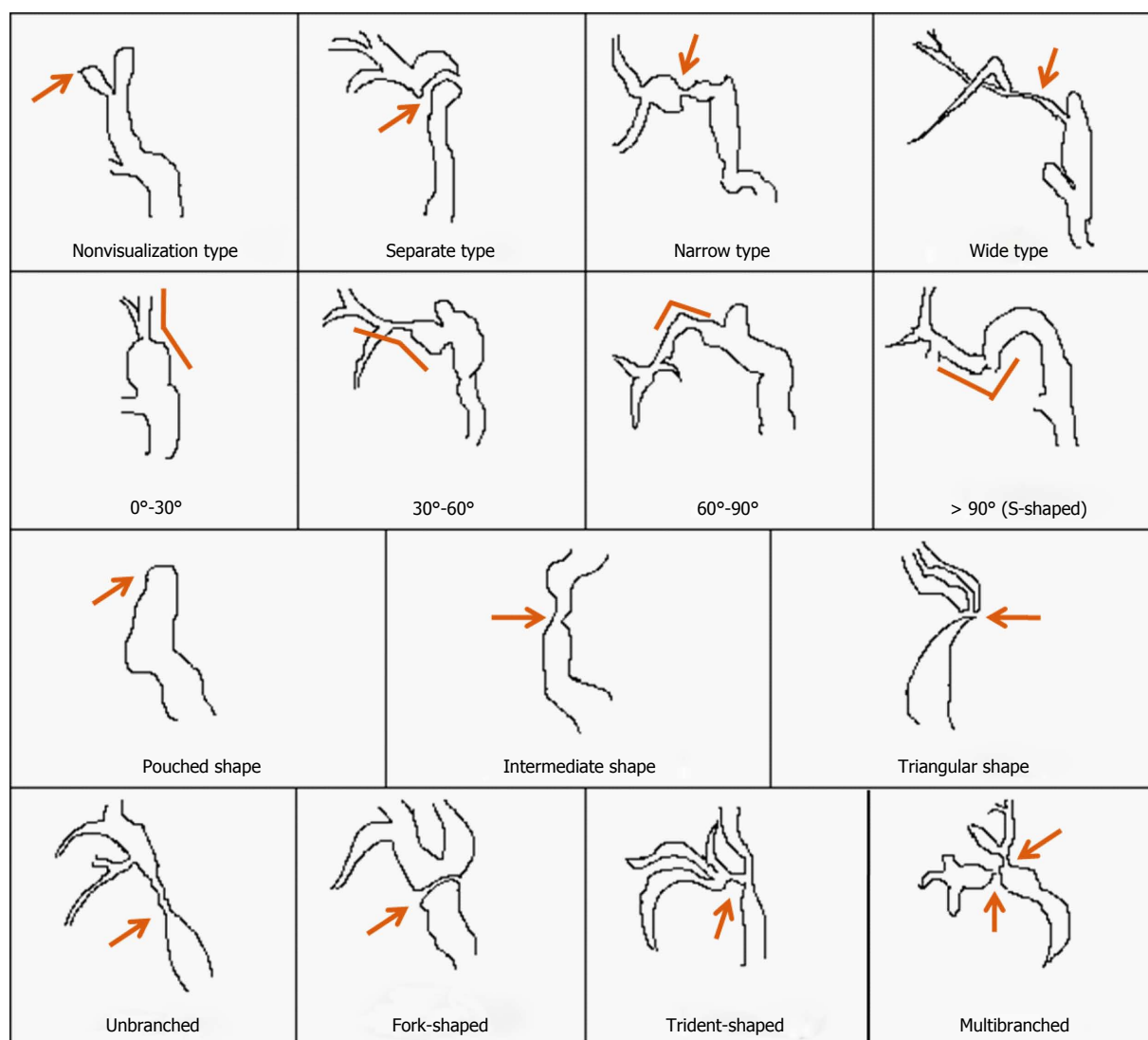


Figure 1 Example of biliary anastomotic stricture after adult living donor liver transplantation.

Types of biliary anastomotic strictures

Several reports have proposed various classifications for dividing the types of biliary anastomotic strictures that occur after an LDLT (Figure 1)^[39]. There is a general consensus that the clinical outcomes and prognoses of the different types of strictures are markedly distinct. As described later, the feasibility and success rate of endoscopic intervention are heavily dependent on the categories of strictures defined on the basis of cholangiography. These may reflect the severity of stricture^[37]. In a recently published study, biliary anastomotic strictures were classified by the morphology of stricture and were divided into the nonvisualization, separate duct, narrow stricture, and wide stricture types^[23]. They also classified the strictures by the angle between the proximal and distal ducts: 0°-30°, 30°-60°, 60°-90°, and > 90° (S-shaped stricture)^[23].

In comparison, some studies divided strictures into three types (pouched, intermediate, or triangular),

based on the shape of the distal-side (donor) of the bile duct anastomosis^[37,40]. One found that initial bile leakage had an important role in the formation of pouched strictures^[37]. Occasionally, the pouched type is named round type, and the triangular type is named tapered type^[23]. Additionally, several Japanese groups divided strictures into four types based on the number of biliary strictures at the proximal side of the biliary anastomosis: unbranched, fork-shaped, trident-shaped, and multibranched (more than three strictures)^[12,25,41]. Interestingly, in right lobe LDLT, most of the biliary anastomotic strictures were fork-shaped or trident-shaped strictures, even if the biliary system had been reconstructed in a single duct-to-duct anastomosis^[12,41]. They proposed that this finding suggested that these biliary strictures arose as a result of ischemic changes extending from the anastomotic site to the proximal biliary tree of the graft^[41]. One study observed a progression of strictures from mild to severe during the period of endoscopic treatment^[22].

Risk factors for biliary complication

Several factors have been identified that can lead to biliary complications after LDLT^[37,38,42]. Ischemic damage, such as hepatic artery compromise, is thought to be the most important factor^[31,41,43]. Further potential ischemic damage is associated with the impairment of peribiliary vascular plexus as a result of prolonged ischemic time or ischemia-reperfusion injury during LDLT. The bile duct epithelium is more vulnerable to anoxic reoxygenation injury than are hepatocytes and the vascular endothelium^[44]. An increased incidence of biliary complications is also associated with technical factors during surgery, which include excessive dissection of periductal tissue, electrocauterization for duct stump bleeding, and tension of the duct anastomosis^[30,31]. Additionally, an organ from an elderly donor^[5], Model for End-stage Liver Disease score greater than 35^[45], routine T-tube placement^[19,31], urgency of transplantation^[24], and immunologic factors such as ABO blood type incompatibility, repeated rejection episodes and chronic rejection^[46] were recognized as risk factors for biliary complications. Some recent studies found a history of bile leakage in the postoperative period to be a significant predisposing factor for stricture development^[5,22,24,43].

Whether the rate of biliary complications is lower in patients undergoing a duct-to-duct choledocho-choledochostomy than in those undergoing a Roux-en-Y choledocojejunostomy has been controversial^[21,47-49]. However, currently it is generally agreed that the type of biliary reconstruction does not affect the development of biliary complication after LDLT^[8,31,50]. The duct-to-duct anastomosis is usually preferred for adult LDLT recipients because it provides the advantages of a shorter operation time, more physiologic bilioenteric continuity, easy endoscopic access to the biliary system, and preservation of the sphincter of Oddi, which avoids reflux of intestinal contents into the bile duct and reduces the risk of cholangitis^[2,5,11,21,37,42,51].

LDLT as a risk factor for biliary complication

Biliary complications are more frequent with transplants from living donors compared with transplants from deceased donors^[18,19,52,53], and these complications occur with a higher frequency in right liver grafts than in left liver grafts^[21]. Increased incidences of biliary complications after LDLT are associated with small diameter and short stump of the anastomotic bile duct, biliary anatomical diversity, complex surgical procedures, occasionally creation of multiple bile duct anastomoses, local ischemia of the peribiliary plexus, and angulated duct anastomosis caused by hypertrophy of the liver graft^[5,8,12,30,42,54,55]. Furthermore, a discrepancy in luminal diameter between the donor and recipient bile duct^[15,56] and the presence of more than one duct orifice in the graft^[8,43,57,58] are significant contributing factors for the development of biliary

complications. Because of these variable difficulties, the process of LDLT itself serves as a risk factor for biliary complications^[4,14,31].

Treatment options for biliary complication

Posttransplant biliary complications occasionally lead to recurring hospital admissions or to graft failure, which necessitates re-transplantation, both of which increase the costs of treatment^[7,15,19,59]. Therefore, early diagnosis and prompt, adequate management of biliary complications have a significant role in determining the recipient's quality of life as well as graft survival^[15,19]. Although no clear therapeutic algorithm has yet been established, many modalities to treat biliary complications have been developed, including endoscopic techniques, percutaneous transhepatic intervention, and surgical procedures^[14,46,48]. The traditional primary approach to management of these conditions in the past was predominantly surgical^[27]. However, with growing expertise, physiologic loads on patients and complication rates related to nonsurgical procedures are acceptably low in comparison with surgical procedures^[11,30,43,60]. At present, nonsurgical approaches have largely replaced reoperation as the initial treatment of biliary complications^[7,11,24,30]. In particular, great developments in endoscopic techniques over the past decade have allowed successful endoscopic access, with demonstrated efficiency in the treatment of the majority of biliary complications^[5,11,12,22,26,46]. Endoscopic treatment is now considered to be the preferred first-line modality for patients that have previously undergone duct-to-duct biliary reconstruction, as it is less invasive, safe, effective, more easily accessible, and more convenient for the patient^[3,5,9,12,23,25,35,38,47,61,62]. Percutaneous transhepatic therapy is then subsequently considered in cases where the endoscopic approach has failed^[3,62]. Surgical revision or conversion from duct-to-duct to Roux-en-Y hepaticojejunostomy anastomosis is very complicated and technically demanding, and is therefore reserved as a rescue therapy when all other modalities have proven unsuccessful^[6,14,15,29].

Endoscopic procedures have proven effective and beneficial in the management of biliary complications after deceased donor liver transplantation (DDLT)^[63-65]. However, it remains controversial whether to apply the same endoscopic procedures to LDLT cases, because LDLT differs from DDLT in the type of graft used^[59]. According to a recent report from the Adult-to-Adult Living Donor Liver Transplantation Cohort Study consortium (A2ALL), although the incidence of biliary complications after LDLT is higher than after DDLT, treatment requirements and time to resolution after development of a biliary complication are similar in LDLT and DDLT recipients. These data refute the common impression that biliary complications after LDLT are a more protracted and less resolvable problem than those occurring after DDLT^[13]. Endoscopic treatment of biliary

complications is equally efficacious in both LDLT and DDLT recipients and should continue to be the first-line of therapy^[35].

Purpose

In this review article, we describe various aspects of endoscopic management of biliary complications after LDLT, including an extensive review of the current literature.

GENERAL PRACTICE OF ENDOSCOPIC MANAGEMENT

An endoscopic technique with endoscopic retrograde cholangiography (ERC) is the primary approach for diagnosing and treating biliary complications after LDLT with duct-to-duct biliary reconstruction. After an overnight fast and conscious sedation, the procedure is performed using a video duodenoscope. The bile duct is selectively cannulated, and a contrast agent is injected through the catheter into the biliary system to obtain a fluoroscopic image. After identification of the type, site, and shape of the biliary complication, based on the completed cholangiographic findings, appropriate therapeutic interventions are performed^[12,18,24,28,37,40,56,59,66]. Conventional therapeutic endoscopy universally involves endoscopic sphincterotomy and cross with a variety of guide-wires, measuring 0.018, 0.025, or 0.035 inches in diameter, through the corresponding lesion, for secure and easy repeated access^[22]. The details of the therapeutic interventions follow below.

Biliary stricture: Anastomotic stricture

If there is an anastomotic stricture, once a guide-wire is traversed into the bile duct proximal to the stricture site, balloon dilatation and endoscopic retrograde biliary drainage (ERBD) stent placement is the current standard treatment^[14,15,22,23,36,47,67,68]. This approach has been demonstrated to be more effective compared with balloon dilation alone^[27,29,63,69]. The balloon is gradually inflated as large as the donor duct size, and single or multiple plastic stents are subsequently inserted. The procedure must be repeated every 3 mo to evaluate the progression of complicated lesions, to dilate the stricture site, to minimize stent occlusion, and to prevent cholangitis or stone formation^[29,47,69]. In addition, an increasing number and larger diameter of stents are progressively replaced at each sequential ERC session to achieve a maximum diameter and greater dilatation^[14,15,36]. There are various protocols for applying this routine technique. A few groups carry out balloon dilatation alone at the first ERC, and if there is residual stricture on follow-up ERC, placing ERBD stents across each stricture^[12]. Recently, more aggressive approaches using maximal balloon dilation and multiple parallel stents, up to the maximum number allowed by the bile duct diameter, with an additional stent placed

adjacent to the first stent, reportedly achieved more expeditious resolution of anastomotic strictures^[70-72]. Some studies suggest trying to insert as many stents as possible at the first ERC^[24,28]. One study suggests rapid-sequence ERC with accelerated dilatation every 2 wk and a shorter stenting duration of an average of 3.6 mo^[72]. In addition, before 4 wk posttransplant, a stent is placed without balloon dilation to avoid anastomotic disruption^[47]. The total duration of stent deployment averages from 6 to 12 mo, with an average of 3 to 4 stent exchange sessions^[19,36,56,63,73]. The treatment in most patients with anastomotic stricture requires balloon dilation of 4 to 10 mm for 30 to 60 s and an ERBD stent of 7 to 10 Fr^[15,19,40,63,72].

Biliary stricture: Non-anastomotic (hilar and intrahepatic) stricture

Endoscopic management is also the first-line modality for non-anastomotic strictures, which is similar to the approach for anastomotic strictures. It includes balloon dilatation of accessible strictures, ERBD stent placement at multiple lesions, and exchange every 3 mo^[14,19,31,32,38,74]. However, the endoscopic treatment of non-anastomotic stricture is more difficult and less satisfactory than that of anastomotic stricture^[12,19,29]. Balloon dilation of all strictures is not feasible because of the multiple diffuse locations of strictures^[74]. The small caliber of the hilar and intrahepatic bile duct may limit the caliber and number of stents placed^[74]. Furthermore, repeated accumulation of biliary sludge or casts gives rise to rapid stent occlusion, recurrent cholangitis, liver abscess, and biliary cirrhosis^[19,32,74]. Patients with non-anastomotic stricture need more frequent and numerous ERC sessions and have a more prolonged time of response^[3,38]. Although non-anastomotic stricture is more resistant and temporarily responsive to endoscopic treatment^[5,36], this endoscopic strategy is able to delay retransplantation and to relieve the symptoms of cholangitis while waiting^[19,74].

Bile leakage

ERC is the gold standard for diagnosis of any kind of bile leakage^[31,36]. When ERC detects the exact site of biliary leakage, early prompt intervention should be performed, because bile leakage is an independent risk factor for the development of stricture^[31]. Bile leakage is successfully treated with transpapillary ERBD stent placement, which bridges and seals the leakage^[14,16,31,32,36]. Although sphincterotomy alone can be effective as a result of reducing pressure in the bile duct, to achieve a satisfactory result, ERBD stent placement typically should be used for diverting bile away from the leakage site^[33]. Whether a bile leak occurs in an anastomotic or non-anastomotic site, the same approach can be used. Although clinical symptoms improve within a few days, complete resolution of the leakage occurs within 5 wk^[5,13,63]. Most centers advocate that the stent should be left in place for about 2 mo, because of delayed healing

owing to the use of immunosuppressive agents^[14,19]. In most cases, a total of 2 ERC sessions is sufficient for treatment of bile leakage^[33]. If there is an associated biliary stricture, the strategy is careful balloon dilatation accompanied by ERBD stent placement beyond both the stricture and the leakage^[14,31]. Bile leakage in a T-tube tract is often self-limiting and may be managed conservatively by leaving the tube open, without further intervention^[31,34,36]. However, if persistent, endoscopic treatment should proceed such that ERBD stent placement occurs parallel to the T-tube, which is removed immediately after ERC^[19].

Biloma

Any kind of bile leakage will result in biloma formation. ERC plays a therapeutic role in defining and eventually treating the underlying bile leakage^[34]. If the associated biloma is symptomatically deteriorative, abundant, or infected, whether intrahepatic or perihepatic, the combination of endoscopic sphincterotomy with or without ERBD stent placement and simultaneous percutaneous catheter drainage is adequate and beneficial^[14,32,36].

Bile duct stones, sludge, casts, debris and other filling defects

Biliary obstruction can also be caused by stones, sludge, debris, or casts after LDLT. The endoscopic management for these is similar to that for non-transplant patients; the obstructions are treated with various combinations of sphincterotomy and balloon retrieval or trapezoid basket extraction^[18,31,35,36]. When biliary stricture is found, it should be treated simultaneously^[19,33]. In the majority of filling defects, especially with stones, management is successfully accomplished in a single ERC session^[14]. However, the endoscopic approach for cast extraction is less favorable for permanent clearance of the biliary tree^[19,34]. In some cases, only a reduction of intraductal pressure by endoscopic sphincterotomy can be sufficient to achieve a favorable outcome. Large balloon dilation of the biliary sphincter orifice (EPBD, endoscopic papillary balloon dilation), with or without sphincterotomy, is reported to be a possible method for the removal of large stones and casts after LDLT, with improved efficacy and minimized complications^[11,12,14].

Sphincter of Oddi dysfunction

Sphincter of Oddi dysfunction is defined as dilatation of the bile duct without stenosis or filling defects, along with biochemical cholestasis^[14,36]. It is assumed that operative denervation of the distal common bile duct causes impaired ampullary relaxation and hypertonic sphincter, which may trigger biliary leakages by increasing the intraductal pressure^[34]. However, it can be also expected to arise from a combination of posttransplant edema and inflammatory stricture due to long-term ERBD stent placement^[19]. Patients are further evaluated with ERC, ideally with manometry^[14,36,65].

Although manometry is essential to confirm the diagnosis, it is rarely performed because of the high risk of post-ERC pancreatitis^[19]. Instead, as long as patients present with symptoms and signs highly suspicious for the condition, endoscopic treatment is initially attempted by endoscopic sphincterotomy, transpapillary stenting, or both^[14,19,33,34,65,75].

SPECIAL TECHNIQUES OF ENDOSCOPIC MANAGEMENT

Endoscopic naso-biliary drainage insertion

Occasionally, instead of an ERBD stent, an endoscopic naso-biliary drainage (ENBD) tube can be used to treat biliary complications, particularly with respect to bile leakage. When bile leakage is confirmed by ERC, ENBD is inserted proximal to the leakage site^[9,11,12,22,27,35]. The ENBD removed after fluoroscopic testing has confirmed resolution of the leakage. Some centers have used ENBD to manage biliary stricture, as a bridge therapy for further inside-stent placement. In case of difficulty in adequate balloon dilatation or biliary stent insertion on the first attempt, ENBD is tentatively placed, followed by replacement with an inside-stent within 1 wk^[11,66,67]. The advantage of ENBD is that it permits frequent ERC follow-up and easy retrieval without the need for additional endoscopic intervention^[19,31]. However, the disadvantages of ENBD stenting are patient discomfort caused by the indwelling tube, prolonged hospital stay, and body fluid loss caused by non-physiologic bile drainage^[19].

Inside-stent placement without endoscopic sphincterotomy

In conventional endoscopic procedures, especially multiple biliary stenting, sphincterotomy is generally performed, because the distal ends of the stents exposed to the duodenum compress the pancreatic orifice, which can lead to acute pancreatitis^[12,41]. However, sphincterotomy induces regurgitation of the duodenal fluid into the graft bile duct and causes reflux cholangitis and frequent stent occlusion^[76]. For these reasons, some groups have employed inside-stent placement without performing sphincterotomy in the treatment of biliary stricture after LDLT^[12,41,67]. The inside-stent is a modified plastic stent placed above the intact sphincter of Oddi^[67]. A distal flap of the stent is removed to facilitate transport into the bile duct, and a nylon thread is attached to the distal side, dropping into the duodenum to permit easy removal^[67]. This procedure provides several benefits, including a lower risk of cholangitis and less frequent stent occlusion with long-term patency, by preserving the function of the sphincter of Oddi^[41,67]. Additionally, as many as three 10 F inside-stents can be placed, because the distal ends of the stents do not compress the pancreatic orifice^[41].

Deep enteroscopy technique: Patient undergoing Roux-en-Y choledochojejunostomy

When posttransplant biliary complications develop in patients who have previously undergone Roux-en-Y choledochojejunostomy or gastric bypass, conventional ERC with a duodenoscope is essentially impossible, because passage of an endoscope through the afferent loop of a Roux-en-Y reconstruction is problematic^[77]. In these cases, a percutaneous transhepatic approach is recommended as the initial treatment modality. However, new developments in deep enteroscopy techniques allow successful endoscopic access to the biliary orifice and anastomosis site^[14,38,77-81]. Initially, the deep enteroscopy technique employed a variable stiffness colonoscope, such as a pediatric colonoscope^[14]. Recently, single-balloon enteroscopy, double-balloon enteroscopy, and spiral overtube-assisted enteroscopy have been used^[14,38]. In double-balloon enteroscopy, a balloon-attached enteroscope is passed through a balloon-attached overtube, and is advanced retrograde through the duodenum, jejunum, and up a Roux limb by alternate inflation of the two balloons^[80]. If applying a spiral overtube, it is installed over the enteroscope. As the spiral overtube is rotated, the small bowel is pulled onto the overtube, eventually allowing the enteroscope to advance through^[14]. Once the biliary anastomosis site is observed, ERC is performed under direct vision through the enteroscope, and adequate therapeutic intervention is subsequently achieved. Several studies have reported the successful balloon dilatation of biliary strictures with the use of a deep enteroscopy technique in patients undergoing Roux-en-Y choledochojejunostomy^[80,82,83]. A few studies support a more invasive approach on endoscopic management for posttransplant biliary complications in patients with an extremely long Roux limb, including performing a percutaneous gastrostomy or jejunostomy tube insertion, followed by enteroscopic access through it^[77,84].

Rendezvous technique

Occasionally there are cases where conventional endoscopic access is unsuccessful. In these failed situations, alternative treatments should be considered to facilitate cannulation of the bile duct. Cannulation of a biliary stricture can be achieved by means of the rendezvous technique, which is a hybrid technique combining percutaneous transhepatic and endoscopic transpapillary approaches^[20,38,81,85-94]. When a guide-wire cannot pass over the stricture by ERC, after performing percutaneous transhepatic cholangiography (PTC) and percutaneous transhepatic biliary drainage (PTBD) catheter placement, a guide-wire is inserted through PTBD tube and is advanced into the duodenum. Once the guide-wire exits the papilla, the wire is captured by the endoscopic Dormia basket, forceps, or snare introduced through ERC, and then is pulled through the biopsy channel of the endoscope^[90]. Through

the guide-wire, the subsequent ERC procedures are followed. This technique is recommended in patients with a sharp or twisted angle at the stricture site^[86,91]. Depending on hospital policy, both parts of the technique can be performed simultaneously in the fluoroscopy unit by both an interventional radiologist and endoscopist^[93], or they can be performed sequentially^[85].

In addition to this classical method, various modified Rendezvous techniques have been attempted. Many have performed a pushing insertion of the guide-wire from the common bile duct into the lumen of a bottle-top metal-tip ERC cannula, instead of capturing the guide-wire with a basket or snare, and then the ERC cannula is advanced over the wire into the bile duct^[20,24,86,90,94]. Another approach uses a Kumpe catheter instead of a guide-wire, because the Kumpe catheter's short length allows for easier manipulation and its slightly angulated end permits easy approximation to the ERC cannula^[20]. Another approach is to use a microcatheter with a smaller wire^[94]. Furthermore, in patients with complete stricture, a modified technique where the capture of guide-wire occurs in the subhepatic space, not in the duodenum, has been performed successfully^[91]. In this approach a guide-wire is inserted *via* ERC, puncturing into the paracholedochal space. The snare is inserted through PTC into the duodenal bulb to catch the guide-wire and pull it through to the outside of the body, establishing bilio-duodenal continuity^[91]. Many studies have demonstrated that the rendezvous technique is useful and safe for the management of biliary stricture after LDLT with duct-to-duct anastomosis^[20,38,86-88,91,94]. Owing to these advances, the Rendezvous technique combined with double-balloon enteroscopy has been introduced for the treatment of biliary anastomotic obstruction after LDLT with Roux-en-Y anastomosis^[81,92]. Some reports support the application of the rendezvous technique for the treatment of bile leakage and biliary anastomotic disruption^[85,89,93]. When a previous ERC or PTC approach to place a stent across the leak site has failed, bile duct continuity can be restored using the modified rendezvous technique, where the grasping of a guide-wire occurs at the biloma^[85,93].

Magnetic compression anastomosis technique

Magnetic compression anastomosis is another hybrid technique, which is used for recanalization of severe biliary strictures after LDLT that cannot be treated with conventional methods^[95-100]. This technique can be applied to completely obstructed or disconnected biliary strictures^[96]. For this procedure, two magnets are introduced on each side of the obstructed bile tract: the first magnet (parent magnet, without wire) is delivered in a transpapillary approach at the inferior site of obstruction through ERC, and the second (daughter magnet, attached with a 30 cm nylon wire) is positioned at the superior site of obstruction through

the PTBD^[99]. The two magnets are approximated to within 2.5 to 4 cm distance under fluoroscopic guidance, if necessary, using a balloon catheter for better advancement^[97]. The two magnets are immediately attracted toward each other, sandwiching the stricture^[100]. The transmural compression of the two magnets causes gradual ischemic necrosis, and thus creates a new anastomosis between the magnets^[6,99]. If a re-anastomosis is successfully formed, the approximated magnets will naturally pass along the bile tract^[97], or else each magnet is respectively pulled out *via* the ERC and PTBD routes^[101]. Finally, after confirming the recanalization, a temporary ERBD stent is positioned across the stricture. Graphic illustration describing the process of magnetic compression anastomosis technique for severe biliary stricture is presented in supplementary material (Supplementary Figure 1). The magnets used for this technique are cylindrical samarium-cobalt rare-earth magnets because of their stronger retention force^[102]. Routinely, the parent magnet (5 mm, 3700 gauss) has a larger diameter and greater strength than the daughter magnet (4 mm, 3200 gauss). In this way the daughter magnet is continuously pulled, and the pair of magnets can easily move into the distal bile duct and intestine, not into the proximal bile duct, once re-anastomosis is established^[99].

The clinical feasibility, safety, and usefulness of the magnet compression duct-to-duct anastomosis technique have been established and demonstrated in various recent reports of severe biliary stricture or obstruction after LDLT^[95-98,100]. Recently, owing to these advances, a number of reports applied the magnetic compression duct-to-enteric anastomosis method for biliary stricture in patients undergoing Roux-en-Y choledochojejunostomy^[99]. They created an anastomosis between the bile duct and the small intestine, using a forward-viewing endoscope or constructing a temporary skin-intestinal fistula to carry the parent magnet near the stricture^[99,101]. Additionally, several technical modifications have been made in the magnetic compression anastomosis technique in recent innovative studies. Some reported the usefulness of prior insertion with a covered, retrievable, self-expanding metallic stent through ERC, where the parent magnet is delivered safely through the stent to the stricture site^[24,95,97,103]. Another pioneer used an overtube with an ERC endoscope to keep the magnet in the initial position while delivering the magnet through the stomach to the bile duct^[97]. They also produce a newly designed magnet with 50% greater magnetic power and a smaller diameter than the previous magnet, to enable to access into narrow bile ducts^[97]. One study reported a case in which a bile duct branch was left without anastomosis and was later successfully anastomosed to the cystic duct stump in a second-look fashion using a magnetic compression anastomosis technique^[103].

Although re-anastomosis depends on the distance between the two magnets and the strengths of the magnets^[97,101], complete recanalization of posttransplant biliary obstruction requires nearly 1 mo^[104]. Nonetheless, this technique can prevent the need for a lifelong external drainage bag and reduce the chance of requiring reoperation for severe biliary stricture after LDLT^[97].

Direct cholangioscopy technique: Single-operator peroral cholangioscopy

When a biliary stricture is severe and too tight to access by a conventional ERC procedure, a direct cholangioscopy technique is valuable for successful guide-wire placement. In particular, the most recent and desirable approach is single-operator peroral cholangioscopy using the SpyGlass® Direct Visualization System (Boston Scientific Corp.)^[14,38,46,75,105,106], in which a single endoscopist operates both scopes with 4-way tip deflection, in contrast to traditional dual-operator cholangioscopy. Recent studies have indicated that single-operator peroral cholangioscopy is feasible and can be successfully performed in LT recipients with biliary complications^[14,75,105-107].

Direct cholangioscopy allows direct visualization of the inner wall of the bile ducts, and a pinhole orifice can be visualized at the stricture site^[38,105]. Under direct cholangioscopic vision, a guide-wire can be passed through the orifice and placed across the tight stricture^[38,105,106]. Direct visualization may also facilitate evaluation of indeterminate biliary strictures or other biliary complications in LDLT recipients requiring ERC^[14,75]. Additionally, direct cholangioscopy enables one to employ advanced intraductal therapeutic maneuvers, such as tissue acquisition for sampling purposes and complete clearance of large or difficult stones, all of which are limitations of conventional ERC techniques using only contrast-mediated fluoroscopic imaging^[14]. A limited number of studies indicate innovative management of biliary stricture guided by single-operator peroral cholangioscopy in LDLT^[105,106]. A recent case report introduced methylene blue-aided peroral cholangioscopy to optically diagnose the ischemic-type of biliary lesions after transplant^[107].

NEW TYPES OF ENDOSCOPIC DEVICES: BALLOONS AND BILIARY STENTS

The selection of an endoscopic treatment method depends on the characteristics of the lesion, including its etiology, location, severity, and findings from ERC imaging. The number, size, and form of the endoscopic devices are determined based on various treatment method options. The increasing development of endoscopic accessory devices, including cannulation catheters, balloons, guide-wires, and stents, will play a significant role in the management of biliary complications after LDLT.

Novel endoscopic balloons

A few preliminary investigations showed that a peripheral cutting balloon is more effective in the treatment of resistant biliary strictures not responsive to standard high-pressure balloon dilatation, with a proven two-year primary patency rate of 55% and secondary patency rate of 78%^[108,109]. Furthermore, the use of paclitaxel-eluting balloons has been introduced as a new treatment option of biliary anastomotic stricture after liver transplant, which achieved a sustained clinical success of 92%^[110]. Paclitaxel, as a mitotic inhibitor, has an antifibrotic effect, and the combination of dilation and antiproliferative therapy is reasonable to resolve biliary strictures characterized by fibroproliferation^[111]. These balloons are known for their safety and efficacy in the treatment of arterial stenosis. Albeit from a preliminary investigation, these innovative results may offer several advantages in the field of LDLT.

Selection of biliary stents

The most commonly used ERBD stent is a plastic (polyethylene) stent. Plastic stents are easy to insert and more cost effective, but have a small diameter and can become clogged over time. Because of the prolonged dilatation and high risk for occlusion, the strategy of multiple side-by-side plastic stents placement has been generally accepted as the standard endoscopic treatment of biliary stricture after LDLT. Despite the excellent outcomes described above, there is often a need for frequent ERC to replace clogged ERBD stents, and repeated ERC interventions can be associated with ERC-related risks, such as pancreatitis, cost, and patient burden^[73].

To reduce the recurrence of biliary stricture and to maintain a longer duration of patency, a metal stent with a larger diameter has been developed^[15]. Traditional metallic open-mesh and uncovered metal stents normally cannot be removed, and are considered a permanently implantable device^[112]. Over time, stent metal penetrates the submucosa of the bile duct, with consequent mucosal hyperplasia and ingrowth that promotes frequent stent occlusion and stone formation^[14,112]. Removal of an embedded stent leads to infection, bleeding, and perforation. Therefore, these stents are typically contraindicated in benign biliary diseases, including posttransplant biliary stricture after LDLT^[25,113-115].

In this setting, the covered, self-expanding metal stent, either partially covered or fully covered, has been introduced^[70]. Because the outer coating of the stents prevents tissue ingrowth into the stent mesh^[14], covered metal stents have less epithelial hyperplasia, less occlusion, and extended patency. Furthermore, it is retrievable. In contrast of uncovered metallic stents, which are difficult to remove and typically require a combination of techniques, removal of a covered metallic stent with a snare is relatively simple and safe,

and can be followed immediately by further endoscopic therapy^[112].

There is an experience in temporary placement of partially covered, self-expanding metal stents to maintain stent patency, with success rate of 94%^[112]. However, the placement of partially covered metal stents, while effective in the initial treatment of biliary stricture, have limited long-term efficacy^[16]. Stent extraction is sometimes difficult or impossible due to the inflammatory reaction in the upper and lower non-covered ends^[70]. As a result, although it is applicable theoretically, the use of partially covered self-expanding metal stents cannot be recommended for therapy of posttransplant biliary stricture.

Instead, a recently developed, fully covered, self-expanding metal stent has emerged as a good alternative in the management of posttransplant biliary complications, especially in patients not responding to standard endoscopic treatment^[14,16,53,70,116-119]. The lack of embedding of the metal into the bile duct wall allows for easier removability overall^[116]. The diameter of this stent is 10 mm, about three times as large as the diameter of the average plastic stent^[70]. The stent is attached to a long retrieval string, and can be removed a couple of months later by grasping the string with a standard forceps^[117]. Several studies have reported that temporary placement of a fully covered, self-expanding, metal stent is feasible and effective in the treatment of refractory biliary stricture after LDLT, showing a success rate of 60% to 87.5%^[14,16,53,70,117,119]. The use of a fully covered, self-expanding metal stent provides a larger stricture dilatation, longer stent patency, fewer ERC sessions and its attendant benefits, such as fewer adverse events, shorter hospital stays, and reduced costs^[16,53,70]. Similar to biliary strictures, other studies have found this stent to be effective in the treatment of persistent bile leakage considered difficult to treat^[14,16]. Although acceptable benefits have been proven, one of the limitations of this stent is the tendency to migrate out of or inside the bile duct, occurring in up to 37.5% of cases^[14,70]. Downstream migration inside the bile duct is a more serious complication. To overcome this disadvantage, a few techniques are suggested, including placement of the stent entirely above the papilla^[14] and use of a modified stent with convex margins and an anti-migrating waist on the central portion^[120]. Currently, the temporary placement of a fully covered, self-expanding, metal stent can serve as a rescue treatment, rather than as a first-line therapy, in patients with biliary complications after LDLT that have failed other management techniques^[119]. In the near future, the use of self-expanding stents made of biodegradable material may further contribute to improved endoscopic therapy for posttransplant biliary complications, through the influence of longer patency, lower biofilm buildup, and an enhanced antiproliferative effect with a single intervention^[104,121].

ENDOSCOPIC MANAGEMENT OF BILIARY COMPLICATION IN DONORS

Biliary complication after LDLT may occur in the donor as well as the recipient. With the increasing number of LDLT, living liver donors are also at increased risk for biliary complications. The most common postoperative complication among donors for LDLT is a biliary complication^[122]. The overall incidence of biliary complications in living liver donors ranges from 2.5% to 15%, with bile leakage being the most common^[6,7,122-128]. In a multicenter study of 393 donors in the United States, 9.2% of donors had bile leakage and 0.5% had biliary stricture^[126]. Biliary complications are seen more commonly with right lobe donation^[122,127,128]. According to a survey in five Asian centers, among 561 right lobe donors, 6.1% had bile leakage and 1.1% had biliary stricture^[128]. In another series of 207 right lobe grafts, 13.0% of donors experienced biliary complications, including a single death after uncontrolled bile leakage^[7]. A national survey in the United States found that 6% of right lobe donors had biliary complications requiring intervention^[129].

ERC is a good modality for diagnosis and treatment of postoperative biliary complications in living liver donors^[127]. The general principles of endoscopic management in donors are similar to those of the recipients, and outcomes are also quite similar. A study of 731 consecutive patients who donated liver grafts for LDLT demonstrated that most donors (80%) with biliary complications were successfully treated by endoscopic treatment^[122].

Bile leakage in donors usually presents within 2 wk of surgery^[32]. Minor bile leakage can be successfully managed with conservative therapy, as leaks resolve spontaneously as long as an adequate surgical drain is placed^[127]. When bile leakage is not cured conservatively, endoscopic management is effective, and should be attempted as the first-line therapy^[122,127]. In one study, 9 of 74 donors (11.2%) had bile leakage, 6 of whom were managed endoscopically with temporary ERBD stent placement, recovering uneventfully^[130]. Another study observed that 7 of 276 donors (2.5%) developed bile leakage, and in 6 of these donors, bile leakage resolved within an average of 15 d after placing an ENBD tube across the site of the leak^[127].

Biliary stricture in donors occurs less frequently compared with recipients^[38], and develops often in donors who had bile leakage immediately after LDLT^[127]. ERC followed by endoscopic balloon dilatation and biliary stent placement is the mainstay of treatment. In one study, all donors with biliary stricture demonstrated a satisfactory improvement by ERBD for an average of 113 d^[127]. Interestingly, biliary stricture can be more difficult to manage after right lobe donation^[32], because the compensatory

hypertrophy and right rotation of the remnant left lobe may play a role in the development of bile duct distortion and deformity^[127]. Some studies found that the angle between the common hepatic duct and the left hepatic duct is more acute in donors with biliary stricture than in those without stricture^[122,127]. A recent report described the use of the magnetic compression anastomosis technique in a donor with biliary stricture after left hepatectomy for LDLT^[131]. In addition, when endoscopic attempts have failed due to inaccessibility to guide-wires, the rendezvous technique may be helpful in the placement of a biliary stent, even in living right liver donors^[89].

ENDOSCOPIC MANAGEMENT OF BILIARY COMPLICATIONS AFTER PEDIATRIC LIVER TRANSPLANTATION

Biliary complications occur among pediatric LDLT, and they are certainly associated with increased morbidity and mortality. Rather, biliary complications are more prevalent in the pediatric transplant population due to the small caliber of the bile duct and vascular structures^[132,133]. Like adult transplant patients, partial liver graft has a higher risk of biliary complication than whole graft in pediatric liver transplantation^[134]. According to a multicenter database from the Studies of Pediatric Liver Transplantation (SPLIT) registry, the incidence of biliary complications within 30 d after pediatric LDLT is 17.5%^[135]. The most common complications are bile leakage and biliary stricture^[132,135,136]. In one series, 33% of pediatric LDLT recipients had biliary complications, and the incidence of biliary stricture and bile leakage is estimated to be 17% and 20%, respectively^[137]. In another recent series, 6.3% of biliary complications overall are observed in pediatric LDLT, with bile leakage and anastomotic stricture occurring in 1.9% and 4.5%, respectively^[138].

In pediatric LDLT, Roux-en-Y choledocojejunostomy is mainly performed for biliary reconstruction because the recipient bile duct is relatively small or because of the presence of underlying liver disease^[24,80,132]. In patients who have biliary atresia and who have had a prior Kasai hepatoportoenterostomy operation, the Roux-en-Y choledocojejunostomy is mandatory^[132]. As a result of this anatomical cause, the biliary tree is inaccessible to endoscopy in most cases^[136] and the success rate of ERC is low^[80]. Although the role of ERC treatment for biliary complications has been demonstrated in adult LDLT cases and is considered first-line therapy^[11,63], therapeutic ERC has not been widely accepted in pediatric LDLT cases^[136]. Recently, however, endoscopic techniques that go beyond previous conventional ERC have been developed, allowing successful endoscopic access. Evolved ERC and endoscopy-based methods can be applied to pediatric patients, thus enabling endoscopic treatment

of posttransplant biliary complications with satisfactory outcomes^[80,81,83,136,139,140].

Some studies described successful enteroscopic balloon dilation of biliary anastomotic strictures after pediatric LDLT with Roux-en-Y choledocojejunostomy by using double-balloon enteroscopy^[80,83,140]. In one of those studies, the rate of the enteroscope reaching the biliary anastomotic sites was 68.0%, and the success rate of enteroscopic balloon dilation was 88.2%^[83]. In these, if anastomotic stricture recurred, enteroscopic intervention was repeated and a biliary stent was placed in all of these patients. Double-balloon enteroscopy has become a less invasive, safe, and effective therapeutic option that permits periodic endoscopic intervention. A novel case reported the rendezvous technique using double-balloon enteroscopy for complete anastomosis obstruction of hepaticojejunostomy after pediatric LDLT: One approach from the bile duct was performed by 2.8-mm-diameter cholangioscopy through a PTBD tube, and the other approach from the jejunum was performed by double-balloon enteroscopy^[81]. Another case highlighted endoscopic treatment with the use of an interventional cardiovascular-based smaller caliber guide-wire and angioplasty balloon in a pediatric LDLT recipient with a biliary anastomotic stricture^[139]. A recent retrospective study demonstrated that ERC was feasible and successful in the diagnosis and treatment of posttransplant biliary complications among pediatric LDLT recipients^[136]. In their ERC procedure, a video duodenoscope was used in pediatric patients with duct-to-duct biliary anastomosis, and a pediatric colonoscope was used for push-enteroscopy in patients undergoing a Roux-en-Y choledocojejunostomy. Following the principles for adult LDLT recipients with biliary complications, minimally invasive and effective ERC treatment can be used in pediatric LDLT recipients whenever endoscopic access to the biliary tree can be obtained^[136].

SUCCESS RATES AND OUTCOME PREDICTORS OF ENDOSCOPIC MANAGEMENT

The treatment of posttransplant biliary complications can be achieved most optimally through diverse endoscopic strategies. The role of endoscopy in this field is unequalled. Currently, the preferred endoscopy method is ERC, followed by therapeutic interventions such as endoscopic sphincterotomy, balloon dilation, stent placement, or stone extraction as indicated. Several studies have recently reported high success rates and factors associated with outcomes in endoscopic management of biliary complications. Table 1 summarizes the results of endoscopic therapeutic options for these biliary complications following adult LDLT. Despite the heterogeneity of the study designs, the evidence shows that endoscopic management is

efficient, guarantees an acceptable clinical outcome, and avoids the need for surgical or percutaneous transhepatic approaches in the majority of patients with biliary complications related to LDLT.

The reported success rate of endoscopic management for biliary anastomotic stricture after LDLT is highly variable, depending on the complicating etiology and technique, and ranges from 64% to 76%^[11,12,16,40,41,43,59]. To be more exact, this rate is the initial technical success rate of the first endoscopic intervention. The final therapeutic success rate of endoscopic treatment ranges from 45% to 93%, with recurrence rates from 13% to 44%, varying according to the follow-up period. Therapeutic success means complete resolution without need for further endoscopic, surgical, or percutaneous procedures for the management of biliary problems. Generally, to achieve resolution of the anastomotic stricture, most patients require multiple ERC sessions, averaging 2.7 to 5.4 per patient, multiple stents of 1.9 to 2.5 per ERC, and stent exchange every 2 to 3 mo^[47,73]. Recurrent strictures are also successfully retreated with the same endoscopic methods. Meanwhile, although non-anastomotic stricture is much less frequently observed after LDLT, the endoscopic management of non-anastomotic strictures achieves a much worse success rate of 25% to 30%, with a higher recurrence rate^[12,29,59,141]. Non-anastomotic stricture is more resistant to endoscopic treatment because of repeated sludge accumulation, frequent and rapid stent clogging, and a resultant demand for multiple procedures. Furthermore, endoscopic dilation and stent placement of multiple hilar and intrahepatic stenosis is technically more difficult^[14]. In contrast, endoscopic methods have better success in the management of bile leakage, with a reported resolution rate of 69% to 100%^[11,18,22,35,58]. This result varied widely depending on whether the bile leaks from a cut surface or from the anastomotic site. According to a recent report from the AZALL consortium^[13], 92% of LDLT recipients with bile leakage resolved their leaks within 6 mo of diagnosis. The median time to tube, stent, and drain-free status after a bile leakage was 1.3 mo. Compared with bile leakage, the probability of resolution of biliary stricture was lower among LDLT recipients. Nevertheless, at 24 mo after diagnosis, 94% of LDLT recipients with biliary stricture were tube, stent, and drain-free.

Despite the high success rates presented, endoscopic intervention in LDLT patients is a technical challenge, mainly because of the complexity of biliary reconstruction^[11,14,31,36]. The bile duct anastomosis in LDLT is small in diameter, more tortuous, sharply angulated, twisted, located proximal to the hilum, and sometimes kinked at the hilar portion, which probably results from fibrosis around the anastomosis and from compensatory hypertrophy of the transplanted liver^[6,43,59]. The small caliber of the donor duct limits the size and number of biliary stents used^[14,59]. The distorted bile duct makes an endoscopic approach

Table 1 Results of endoscopic management of biliary complications after adult living donor liver transplantation: A review of the literature

Ref. ¹	Type of biliary complication	Initial technical success, <i>n</i> (%) ²		Final therapeutic success, <i>n</i> (%) ³		Recurrence at a mean follow-up months, <i>n</i> (%) ⁴	Endoscopic treatment modality	Number of ERC session per patient ⁵	Duration for final success (mo) ⁵	Factors affecting endoscopic treatment outcomes
Hisatsune <i>et al</i> ^[41]	D-D	14/22	(63.6)	2/14	(14.3)	-	IS	1	12	Number of the proximal duct at D-D
Shah <i>et al</i> ^[35]	D-D	3/4	(75.0)	3/3	(100)	-	BD and/or PS	1.5	2.3	
	Leakage	8/8	(100)	8/8	(100)	-	PS with/without ES	2	1.4	
Zoepf <i>et al</i> ^[26]	D-D	7/12	(58.3)	7/7	(100)	1/7 (14.3) at 10.0	BD and/or PS	3.5	8	
Yazumi <i>et al</i> ^[112]	D-D	48/75	(64.0)	28/55	(50.9)	3/28 (10.7) at 1.8	IS with/without BD	-	9	Crane-neck deformity
	Leakage	13/16	(50.0)	8/13	(61.5)	-	ENBD	-	0.6	Non-anastomotic stricture
Tsujino <i>et al</i> ^[111]	D-D	12/17	(70.6)	9/12	(75.0)	4/9 (44.4) at 10.1	BD with ENBD/IS	4.1	-	
Lee <i>et al</i> ^[22]	D-D	12/17	(70.6)	7/14	(50.0)	0/7 (0.0) at 13.1	BD with ENBD/PS	3.9	7.2	Stricture
	Leakage	11/13	(84.6)	9/11	(81.8)	0/9 (0.0) at 13.1	PS or ENBD	2.2	3	Sharp angulation of D-D
Tarantino <i>et al</i> ^[16]	D-D	14/20	(70.0)	7/14	(64.3)	-	BD with double PS	3.4	-	Concomitant bile leakage
	Leakage	11/13	(33.3)	3/4	(75.0)	-	ES with PS	2.3	-	Continuous bile leakage despite PS
	Both	4/6	(66.7)	0/4	(0.0)	-	-	-	-	Persistence of stricture after 1 yr
Kato <i>et al</i> ^[59]	D-D	31/41	(75.6)	28/35	(80.0)	7/28 (25.0) at 9.3	BD with PS	4	14.5	Concomitant bile leakage
Kim <i>et al</i> ^[40]	D-D	-		38/60	(63.3)	5/38 (13.2) at 7.9	BD with PS	3	-	Shape of distal duct at D-D: pouched
Kobayashi <i>et al</i> ^[66]	D-D	-		7/16	(43.8)	-	-	-	-	Delayed diagnosis of stricture
Seo <i>et al</i> ^[43]	D-D	15/26	(57.6)	20/29	(68.9)	6/20 (30.0) at 28.0	BD with PS	2.3	6.8	Late onset over 24 wk
										Delayed diagnosis of stricture
										Short duration of biliary stenting
Gómez <i>et al</i> ^[18]	D-D	4/10	(40.0)	2/4	(50.0)	-	BD with/without PS	2	-	
	Leakage	3/4	(75.0)	3/3	(100)	-	ES with/without PS	3	-	
Chang <i>et al</i> ^[28]	D-D	63/101	(62.4)	48/90	(53.3)	-	BD with PS	3.2	11	Non-anastomotic stricture
Lee <i>et al</i> ^[23]	D-D	64/137	(46.7)	38/68	(55.9)	-	PS or ENBD	4.8	-	Hepatic artery stenosis
										Stricture-to-ERC interval
										Morphology of stricture : narrow, separate duct, nonvisualized
										Shape of distal duct at D-D: round tip
Kim <i>et al</i> ^[61]	D-D	82/147	(55.8)	52/141	(36.9)	6/52 (11.5) at 21.1	BD with PS	6.3	12.7	Early onset within 1 yr after LDLT
Yaprak <i>et al</i> ^[58]	D-D	-		7/13	(53.8)	-	-	-	-	Long length of stricture
	Leakage	-		5/7	(71.4)	-	-	-	-	More than 1 bile duct anastomosis
Chan <i>et al</i> ^[142]	D-D	8/8	(100)	6/8	(75.0)	0/6 (0.0) at 3.0	BD with PS	4.7	4.2	Disuse of intraoperative biliary stent
Kurita <i>et al</i> ^[67]	D-D	94/118	(79.7)	81/92	(88.0)	8/81 (9.9) at 53.0	BD with IS	1.4	6.3	Use of ES
Hsieh <i>et al</i> ^[71]	D-D	32/38	(84.2)	38/38	(100)	8/38 (21.1) at 9.45	BD and maximal PS	4	5.3	Right lobe liver graft
										High-grade stricture
										Sharp angulation of D-D
										Conventional PS <i>vs</i> maximal PS
Chok <i>et al</i> ^[37]	D-D	-		41/56	(73.2)	-	BD with/without PS	3	-	Younger recipient age
										Longer operation time
										Shape of distal duct at D-D: pouched
										Initial bile leakage

Na <i>et al</i> ^[24] D-D	Stricture	53/65 (81.5)	112/129 (86.8)	-	BD and maximal PS	3.2	-	Early period of transplant experience
Chang <i>et al</i> ^[86] D-D	Stricture	20/20 (100)	59/64 (92.2) 13/20 (65.0)	-	Rendezvous Rendezvous	- 2	- 7.2	Sharp or twisted angle at stricture
Mita <i>et al</i> ^[77] C-J	Stricture	7/22 (31.8)	-	0/7 (0.0) at 13.3	BD (deep enteroscopy)	2	2.5	Tube jejunostomy in long Roux limb
Kamei <i>et al</i> ^[82] C-J	Stricture	5/9 (55.6)	7/9 (77.8)	4/7 (57.1) at 27.6	BD (deep enteroscopy)	2.1	-	Use of a single ERC intervention
Jang <i>et al</i> ^[97] D-D	Stricture	10/12 (83.3) : magnet approximation	10/10 (100) : recanalization 9/10 (90.0) : stent-free	1/9 (11.1) at 3.3	Magnetic compression anastomosis	- 6.1	2.5	Length of stricture LDLT-to-ERC interval Architecture of the bile duct Strength of the magnet

¹References: Author, Type of biliary reconstruction in study population; ²Initial technical success, *n* (%): patients who underwent successful ERC intervention/patients receiving a first session of ERC (excluding prior transhepatic rendezvous); ³Final therapeutic success, *n* (%): patients who achieved cholangiographic resolution without need for further endoscopic, surgical, percutaneous procedures (e.g., stent-free) / patients treated by ERC interventions; ⁴Recurrence, *n* (%): patients with recurrent biliary problems with cholangiographic evidence and the need for subsequent intervention/patients who resolved; ⁵Values are expressed as mean. BD: Balloon dilatation; C-J: Biliary anastomosis with Roux-en-Y choledochojejunostomy; D-D: Duct-to-duct biliary anastomosis; ENBD: Endoscopic nasobiliary drainage; ERC: Endoscopic retrograde cholangiography; ES: Endoscopic sphincterotomy; IS: Inside-stent placement above the intact sphincter of Oddi; PS: Plastic stent placement.

difficult. Several studies have reported that the most common reason for failure of endoscopic treatment is the inability to pass the guide-wire through the anastomotic site^[12,41,43,54,61,142]. Moreover, in cases with multiple duct anastomosis or ductoplasty, it may be very difficult to navigate each branch with a guide-wire^[59]. In case of anastomosis constructed near the hilum with a short distance from the second branch, guide-wire passage is infeasible^[43,59].

Recently, numerous studies have identified factors predicting failure of primary ERC interventions. It is a foregone conclusion that endoscopic intervention will be unsuccessful for non-anastomotic strictures or ischemic biliary lesions accompanying hepatic artery complications^[12,28]. One study reported that repeat surgery for a non-biliary indication in the first posttransplant month is a predictor of endoscopic management outcome, since that is potentially related to ischemia^[47]. Concomitant bile leakage also contributes to ERC failure^[16,22,59]. When bile leakage is present, the anastomotic site may be obscured by the leaking of contrast material, precluding passage of a guide-wire^[59]. The LDLT-to-ERC interval or stricture-to-ERC interval has an impact on ERC failure^[23,43,61]. The rate of failure of primary ERC therapy is high in patients with late onset and delayed diagnosis of biliary stricture after LDLT. Many studies have demonstrated that failure of a primary ERC is associated with cholangiographic findings, such as the morphology of the stricture, shape of the distal duct tip, and the angle between the proximal and distal bile ducts^[12,22,23,25,37,40]. Narrow strictures or separate duct type strictures have a higher failure rate than do wide strictures. In nonvisualized strictures, endoscopic intervention often fails^[23]. Pouched (round tip) distal strictures are the most difficult type to manage with endoscopic intervention^[23,37,40]. Sharp angulation of

the anastomotic bile ducts is also a reported cause of ERC failure^[12,22]. The most representative example is the crane-neck deformity, in which cholangiography shows a sharp angulation of the anastomotic stricture, characterized by a severely bent common bile duct that looks like a crane's neck^[12]. In patients with an anastomotic stricture with a crane-neck deformity, because the biliary anastomosis is located far below the highest portion of the duct, endoscopic intervention is unsuccessful. Additionally, a study reported that strictures recur more frequently in patients with a shorter duration of stenting^[43]. A report from the A2ALL consortium revealed that increased experience, with more than 15 biliary complications at a center, is directly associated with a significantly shorter time to resolution, indicating a learning curve for endoscopic management^[13].

CONCLUSION

Despite consistent improvements in the overall outcomes of LDLT donors and recipients, the bile duct is still the most common site for postoperative complication, the so-called the Achilles' heel of LDLT^[143]. Biliary complications after adult as well as pediatric LDLT occur commonly in both donors and recipients, and can lead to significant morbidity and even mortality unless successfully treated. With the majority of patients requiring long-term and repeated therapies, these make the management of biliary complications a major distress during the postoperative follow-up of donors and recipients. At present, these complications can be definitively treated and optimally managed through various endoscopic procedures, including sphincterotomy, balloon dilatation, multiple stent placement, and filling defects extraction. Although the outcome of endoscopic management depends on both

the etiology and location of the biliary complication, several recently published reports clearly demonstrate the safety, long-term efficacy, and superior outcomes of endoscopic therapy for biliary complications after LDLT. Recent technological developments, such as deep enteroscopy, direct cholangioscopy, magnetic compression, or removable fully covered, self-expanding metal stents, now allow for more transpapillary access and a better stenting effect. These developments are progressively expanding the scope and role of therapeutic endoscopy in LDLT patients with biliary complications. Based on these results, therapeutic endoscopy is recommended as a standard first-line approach, and percutaneous transhepatic and surgical modalities may serve as subsequent rescue procedures in failed or resistant cases of endoscopic therapy. In the future, more effective new endoscopic techniques with refined accessory devices will become available and be established to increase optimal results.

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Aberrant post-translational protein modifications in the pathogenesis of alcohol-induced liver injury

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Abstract

It is likely that the majority of proteins will undergo post-translational modification, be it enzymatic or non-enzymatic. These modified protein(s) regulate activity, localization and interaction with other cellular molecules thereby maintaining cellular hemostasis. Alcohol exposure significantly alters several of these post-translational modifications leading to impairments of many essential physiological processes. Here, we present new insights into novel modifications following ethanol exposure and their role in the initiation and progression of liver injury. This critical review condenses the proceedings of a symposium at the European Society for the Biomedical Research on Alcoholism Meeting held September 12-15, 2015, in Valencia, Spain.

Key words: Alcohol; Acetylation; Liver; Carbonylation methylation; Dysfunction; Methylation; Glycosylation; Phosphorylation; Ubiquitination; Sumoylation; Betaine; Post-translational protein modification

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Core tip: A majority of proteins in our body undergo orchestrated post-translational modifications that influence protein structure and function. Chronic ethanol administration causes aberrant post-translational modification of proteins that play a critical role in the pathogenesis of alcoholic-induced liver damage.

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INTRODUCTION

Ethanol consumption leads to many adverse functional and structural molecular changes in multiple organs and accounts for 2.5 million deaths globally each year^[1,2]. Ethanol is mainly metabolized in the liver; this organ is therefore most susceptible to its toxic effects^[3,4]. Sustained alcohol misuse produces a wide spectrum of hepatic lesions, the most characteristic being steatosis, hepatitis and fibrosis/cirrhosis^[5]. Steatosis, characterized by fat accumulation in hepatocytes, develops in 90% of individuals who drink more than 16 g of alcohol per day^[6]. In 30%-40% of individuals reporting chronic alcohol abuse, there is development of hepatitis which is characterized by inflammatory changes in the liver and ballooning degeneration of hepatocytes. In later stages of alcoholic liver disease (ALD), collagen deposition and regenerative nodules can result in the development of fibrosis and cirrhosis, respectively^[2]. Better understanding of the mechanisms by which alcohol damages the liver may yield new pharmacologic strategies to blunt, halt, or reverse disease progression, potentially even in inveterate alcoholics.

Hepatic dysfunction due to chronic ethanol consumption is multifactorial involving dysregulation of multiple cellular pathways^[7-9]. Significant to the aforementioned dysregulation is abnormal post-translational modification of proteins^[10]. About 50%-90% of proteins in our body undergo orchestrated post-translational modifications that could influence protein structure and function. These modifications may be enzymatic or non-enzymatic and play an important role in functions of proteins through the regulation of activity, turnover and localization and/or interactions to maintain cellular hemostasis. These modifications include phosphorylation, acetylation, methylation, glycosylation, ubiquitination, sumoylation

and ISGylation. Directed or inadvertent exposure to stressful factors, including chronic ethanol exposure, has been shown to cause aberrant post-translational modifications. Additionally, bioactive products of enzymatic or non-enzymatic lipid oxidation may also cause protein modifications with potential functional damage to protein^[11] as well as impact the function of various metabolic pathways. This brief overview will focus on recent advances that have been made using global proteomic approaches and bioinformatics to better understand the impact of these altered post-translational modifications by ethanol.

Alcohol consumption significantly alters several post-translational modifications of proteins and this has been reviewed recently^[10]. Here, we review the newly described and pathogenically relevant alterations that play important roles in the progression of ALD. First we discuss the undesired post-translational modifications that may occur *via* electrophilic species including reactive aldehydes (carbonylation), acyl groups (acetylation) and sugar moieties (glycosylation).

CARBONYLATION

A key contributor to the pathogenesis of ALD is enhanced hepatocellular oxidative stress resulting from the production of reactive oxygen species *via* induction of Cyp2E1 as well as xanthine and NADPH oxidases^[12-16]. These reactive species, in turn, induce lipid peroxidation of unsaturated fatty acids including linoleic acid forming α/β unsaturated aldehydes^[17,18]. The best characterized of these carbonyl-derivatives include 4-hydroxy-2-nonenal (4-HNE), 4-oxo-2-nonenal, malondialdehyde (MDA) and acrolein. Following their formation, these highly reactive lipid electrophiles modify DNA as well as lysine, cysteine and histidine residues on proteins, thereby impairing their structural or catalytic capabilities. Early proteomic approaches to identify carbonylated proteins in ALD used 2-dimensional electrophoresis followed by protein identification. These techniques were not very sensitive and only a handful of proteins were identified^[19,20]. A commonality of all these proteins was the fact that all were very highly expressed, which permitted easier identification. Of interest, the majority of identified proteins were involved in either protein folding (heat shock proteins) or hepatocellular oxidative stress responses.

Recent advances in biotin hydrazide chemistry and in the sensitivity of mass spectrometry have allowed for a more in depth proteomic approach to identify less abundant proteins modified by reactive aldehydes in ALD. To date, using global proteomic approaches, over 2000 proteins that undergo carbonylation have been identified in either murine models or in human hepatic tissue isolated from patients with end-stage ALD^[21-23]. Using enriched cellular fractions, chronic ethanol consumption led to an increase in carbonylation of microsomal and

cytosolic proteins. Comprehensive pathway analysis of identified proteins revealed that ethanol consumption impacted many different cellular pathways foremost of which are the fatty acid metabolic, tricarboxylic acid cycle and amino acid metabolism. By increasing carbonylation of proteins involved in these pathways, mechanistic links have been proposed for ethanol's impact on lipid accumulation as well as how acetyl CoA contributes to nutritional imbalances evident in alcoholics. These findings are further supported by an additional study that examined the effects of deletion of glutathione S-transferase A4-4 (GSTA4-4) which functions to remove 4-HNE reducing the effects of reactive aldehydes^[24]. Using GSTA4-4 knockout mice and employing proteomics approaches, it was determined that carbonylation was increased in mitochondrial fractions especially in pathways regulating oxidative stress, fatty acid metabolism and amino acid metabolism supporting the contribution of GSTA4-4 in protecting mitochondria from reactive aldehydes (Supplementary Table 1). Concurrently, we have reported that carbonylation is increased in tissue obtained from end-stage alcoholics^[23]. Not surprisingly, following mass spectral analysis, increased carbonylation of proteins regulating oxidative stress, metabolic and cytoskeletal processes were increased^[24].

GLYCOSYLATION

In cells, glycosylation of proteins contributes to numerous cellular functions including assisting in proper protein folding as well as cell to cell adhesion. Global proteomic approaches and 2-dimensional electrophoresis were performed on microsomal fractions consisting primarily of smooth and rough endoplasmic reticulum, isolated from chronically ethanol fed mice. These studies revealed a significantly decrease in microsomal glycosylation following 8 wk of alcohol consumption. Subsequent bioinformatic pathway analysis revealed significant decreases in glycosylation of proteins regulating protein folding, redox homeostasis and the unfolded protein response among others. These results suggest that decreased glycosylation may contribute to the observed increased in ubiquitinated proteins in murine models of ALD^[25].

ACETYLATION

In hepatocytes, acetylation of lysine residues results in regulation of many cellular functions including gene expression and metabolism. As it is metabolized, ethanol is converted to acetaldehyde by alcohol dehydrogenases followed by removal of the aldehyde group by mitochondrial aldehyde dehydrogenases (ALDH2) to produce acetate, which is converted to acetyl CoA^[26]. By way of protein acyl transferases, acetyl CoA is then utilized in part as a substrate for

protein acetylation. Therefore, it is not surprising that over the last decade, ethanol abuse has been determined to directly affect protein acetylation^[27-30]. In murine models of ALD, ethanol decreases expression of the class III nicotinamide adenine dinucleotide (NAD⁺/NADH) dependent protein deacetylases, Sirtuin 1 (SIRT1) and Sirtuin 5 and decreases enzymatic activity of Sirtuin 3^[31-35]. All these changes combined decrease the overall cellular deacetylase activity that ultimately results in an increase in protein acetylation. Using traditional Western blotting and immunoprecipitation techniques chronic ethanol induces hyperacetylation of several key metabolic regulators, including PPAR γ co-activator 1 α , sterol regulatory binding element protein 1 (SREBP-1c) and forkhead transcription factor 1.

In recent experiments, mass spectrometry and proteomic approaches have been applied to identify proteins and pathways that are acetylated following chronic ethanol consumption. Using whole cell extracts and matrix-assisted laser desorption mass spectrometry, Shepard *et al.*^[30] identified 40 proteins that are acetylated following chronic ethanol administration. Pathway analysis revealed that of these 40, the majority was predominantly mitochondrial proteins and there was a significant preference for proteins regulating lipid metabolism as well as oxidative stress^[29,30]. More recently using mitochondrial enriched fractions isolated from ethanol-fed wild-type and SIRT3 knockout mice, we determined that chronic ethanol impacted acetylation of proteins regulating lipid metabolism, oxidative stress, as well as mitochondrial pathways including amino acid biosynthesis and the electron transport chain^[32].

SUMOYLATION

A member of the ubiquitin family, SUMO, comprised of four distinct proteins in humans (SUMO-1, -2, -3 and -4), is receiving growing interest since its discovery less than a decade ago^[36]. SUMO-4 shows similarity to -2/3 but it is as yet unclear whether it is a pseudogene or merely restricted in its expression pattern^[37]. The sumoylation cycle is a multistep process, involving maturation, activation, conjugation and deconjugation, and regulates the function and fate of a large number of proteins involved in many cellular pathways including transcription, intracellular transport, DNA repair, replication, and cell signaling^[38,39]. Sumoylation, as an enzymatic cascade, resembles that of ubiquitination, including an ATP dependent step, the E1-activating enzyme Aos1/Uba2 (SAE1/SAE2) forms a thioester bond between its catalytic cysteine (Uba2 C173) and the C-terminal carboxy group of mature SUMO. From there, SUMO is transferred to the catalytic cysteine (C93) of the E2-conjugating enzyme (Ubc9). In the last step of this cascade, an isopeptide bond is formed between SUMO and the 3-amino group of a lysine side chain. Specific isopeptidases, members of the SENP family, ensure reversibility of this modification^[40,41].

Sumoylation is often increased under oxidative stress^[42]. Recent reports demonstrate that ubiquitin conjugating enzyme 9 (Ubc9), the sole E2 enzyme of sumoylation, is induced in ethanol treated mice^[43]. However, the functional significance of this finding remains unknown. However, a number of sumoylated proteins have been identified in the liver after ethanol administration and other injury models. A notable example is the enzyme methionine adenosyltransferase II α (MAT α 2) which has been shown to increase upon ethanol exposure^[44] is sumoylated. This modification likely plays a critical role in its stability^[45].

Nrf2, a well-characterized transcription factor is known for its role in activating anti-oxidant response element (ARE), forms heterodimers with small Maf (MafG, MafK and MafF) proteins. We recently reported that Nrf2 and MafG are sumoylated and this facilitates their heterodimerization and trans-activation of the ARE in activated hepatic stellate cells^[46].

Increased levels of lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, is frequently found in cirrhotic patients^[47] and is observed to lower glutathione (GSH), a potent anti-oxidant. GSH is highly concentrated in the liver and synthesized in the cytosol in a tightly regulated manner. Key determinants of GSH synthesis are the availability of the sulfur amino acid precursor, cysteine, and the activity of the rate-limiting enzyme, glutamate cysteine ligase, which is composed of a catalytic and a modifier (GCLM) subunit. LPS inhibits the sumoylation machinery suppressing the expression of the sole E2 enzyme Ubc9. This results in reduced Nrf2 and MafG sumoylation affecting their heterodimerization and trans-activation of the ARE present in GCLC and GCLM in macrophages and hepatocytes^[48].

Although considerable progress has been made in the identification of sumoylated proteins and the characterization of the effects of the modification of these particular substrates, little is known in regards to the global regulation of SUMO conjugation in ALD.

ISOASPARTYL DAMAGE

Ethanol consumption specifically triggers a unique protein post-translational damage as isoaspartate peptide linkages in proteins^[49]. This protein isoaspartate damage is due to an inhibition of the protein repair enzyme, protein isoaspartyl methyltransferase (PIMT).

PIMT normally acts to resist the accumulation of isoaspartate damage that arises through protein aging, and as a consequence of oxidative damage to proteins^[50]. PIMT is a methyltransferase that utilises S-adenosylmethionine (SAM) as a methyl donor. PIMT methylates isoaspartate residues in peptides and proteins, a process that triggers isoaspartate elimination and restoration of protein function. One of the detrimental actions of ethanol consumption is impaired methionine synthase-

catalysed remethylation of homocysteine to generate methionine, the metabolic precursor of SAM. A subsequent depletion of SAM availability limits the activity of SAM-dependent methyltransferases, such as PIMT. This inhibition of methylation reactions is further exacerbated by the ethanol-induced increase in the level of S-adenosylhomocysteine (SAH), a potent inhibitor of numerous SAM-dependent methyltransferases including PIMT^[49,51-54].

Animal studies have demonstrated the benefits of dietary supplementation with betaine, a pro-methylating agent, to counter some of the ethanol-induced changes in the metabolite levels of the methionine metabolic pathway^[49,51-54]. Rats fed a control or ethanol liquid diet (36% of calories) for a period of 4 wk with or without dietary supplementation with 1% betaine revealed that the ethanol-induced reduction of the methylation potential (*i.e.*, the lowering of the hepatocellular SAM:SAH ratio to approximately 43% of control level) was rectified in rats fed an ethanol diet supplemented with betaine^[49,51-54]. Concomitant with the changes in the SAM:SAH ratio, the ethanol-induced increase of damage to cellular proteins as isoaspartate was also alleviated by betaine supplementation^[49,51-54].

A proteomic approach was adopted to investigate the mechanism by which betaine was able to alleviate the ethanol-induced increase of isoaspartate damage. One dimensional and two dimensional protein separations and differential protein staining techniques revealed that betaine supplementation increased the expression of betaine homocysteine methyltransferase-1, methionine adenosyl transferase-1 and glycine N-methyltransferase, and these enzymes act to collectively increase SAM levels and normalise the SAM:SAH ratio^[55].

To further investigate the influence of the SAM:SAH ratio on PIMT activity and cellular isoaspartate damage, primary hepatocytes taken from control or ethanol-fed rats were cultured. Cells were incubated *in vitro* with tubercidin or adenosine, agents that elevate cellular SAH levels^[56]. These agents produced an additive increase of isoaspartate damage to that detected from ethanol consumption, indicative of an additional lowering of the SAM:SAH ratio and further inhibition of PIMT activity.

To identify liver protein target(s) of PIMT that accrue isoaspartate damage after ethanol consumption, proteins from control and ethanol fed rats were exogenously methylated using PIMT and ³H-SAM methyl donor. Novel, sensitive autoradiographic imaging^[57] was used to reveal increased isoaspartate methylation at liver protein bands of 75-80 kDa, 95-100 kDa, and 155-160 kDa. Column chromatography used to enrich isoaspartate-damaged liver proteins indicated that damaged proteins from ethanol-fed rats mirrored those that accumulate in the livers of PIMT knockout mice. The about 160 kDa protein target of PIMT was further purified and

fractionated, and identified as carbamoyl phosphate synthase-1 (CPS-1)^[58]. This is a mitochondrial enzyme that catalyses the synthesis of carbamoyl phosphate from ammonia and bicarbonate, and is the first and rate-limiting step of the urea cycle. Resolution of liver proteins by one dimensional polyacrylamide gel electrophoresis and Coomassie blue staining also showed that cytosolic CPS-1 protein levels increased by approximately 20% in rats administered ethanol for 4 wk. A subsequent study of ethanol administration for 8 wk showed that the levels of cytosolic CPS-1 now increased approximately 2-fold over those of control animals; indicating that cytosolic CPS-1 levels correlated with the duration of alcohol consumption. Increased cytosolic CPS-1 was also detected in PIMT knockout mice compared to their control littermates. This release of liver CPS-1 into the cytosol as a response to ethanol consumption or in PIMT knockout mice is presumed to reflect mitochondrial damage and redox stress.

These studies highlight the accumulation of atypical isoaspartate-containing abnormal proteins following chronic ethanol exposure. The animal studies employed, however, are of relatively acute alcohol administration, and it is hypothesised that in alcoholic patients sustained and cumulative isoaspartate protein damage across a broad number of target proteins would ensue and contribute to liver cell damage and pathology.

METHYLATION

Here, we will discuss the role of this post-translational modification in the regulation of innate immunity in hepatitis C virus (HCV) infection combined with ethanol exposure. About 3% of world population is infected with HCV, the most common blood-borne infection in the United States. By 2010, 2.7-3.9 million people were diagnosed with chronic HCV-infection, and there are about 17000 new cases of acute infection per year. Hepatitis C and alcohol are the most widespread causes of liver disease worldwide, and approximately 80% of patients with a history of Hepatitis C and alcohol abuse develop chronic liver injury^[59]. Almost one-third of alcoholics with clinical symptoms of liver disease have been infected with HCV, which is four times the rate of HCV infection found in alcoholics who do not have liver disease. Alcohol consumption in HCV-infected patients exacerbates liver disease leading to rapid progression to fibrosis, cirrhosis and even hepatocellular carcinoma^[60]. Alcohol-consumption reduces responsiveness of HCV patients to anti-viral treatment; only 7% of heavy drinking HCV patients are responders to interferon therapy^[61]. However, despite the direct acting anti-viral agents (DAA) changing the treatment backbone of HCV infection from IFN-ribavirin, the effectiveness of DAA would also depend on the endogenous IFN α -mediated activation

of antiviral genes in HCV-infected hepatocytes.

The mechanism by which alcohol consumption exacerbates the course of HCV progression is not clear. Since HCV and alcohol alter innate immunity in hepatocytes, there is a strong possibility that their synergistic effect on innate immunity contributes to HCV spread and progressive liver injury. Activation of an anti-viral innate immune response is based on IFN signaling, which requires activation of IFN-sensitive genes (ISG) *via* the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway. Transduction of IFN α signal requires phosphorylation of STAT1 and STAT2. The attachment of STAT1 to DNA becomes possible if STAT1 is methylated on arginine residue(s). It has been shown that HCV subverts the IFN α -mediated JAK-STAT signaling through the reduction of intrahepatic STAT-1 and -2 phosphorylation^[62] and reduces STAT-1 methylation leading to suppression of ISGs^[63,64].

Ethanol also is known to suppress methylation reactions leading to impaired methylation of multiple proteins and enzymes^[49,51-54]. We hypothesize that ethanol potentiates HCV-mediated impairment of methylation-regulated IFN signaling in liver cells, thereby decreasing antiviral protection in liver cells. When HCV-infected Huh7.5-CYP2E1 cells were exposed to an extracellular system that continuously generates the ethanol metabolite, acetaldehyde (Ach) in physiological quantities, STAT1 methylation was suppressed on both arginine and lysine residues^[65]. Suppression of STAT1 methylation is regulated by protein arginine N-methyltransferase 1 and lysine methyltransferases, and can be induced by specific methyltransferase inhibitors. The effects of Ach on STAT1 methylation are protein phosphatase 2 dependent. The impaired methylation of STAT1 increases the complex formation between STAT1 and the pathway inhibitor, protein inhibitor of activated STAT-1 (PIAS1), preventing the attachment of IFN-activated STAT1 to DNA followed by antiviral gene activation. This mechanism is schematically presented as Figure 1. Methylation-dependent dysregulations of IFN signaling in hepatocytes were attenuated by supplementation with the pro-methylating agent, betaine^[65].

Thus, Ach potentiates the ability of HCV to down-regulate activation of ISGs by interferon and plays a pivotal role in methylation-dependent suppression of innate immunity in hepatocytes, which are primary sites of both HCV replication and ethanol metabolism.

OXIDIZED METABOLITES OF FATTY ACIDS

Alcohol administration results in increased production of enzymatic or non-enzymatic lipid oxidation products, which may also cause protein modifications and potential functional damage to proteins^[11] as well as

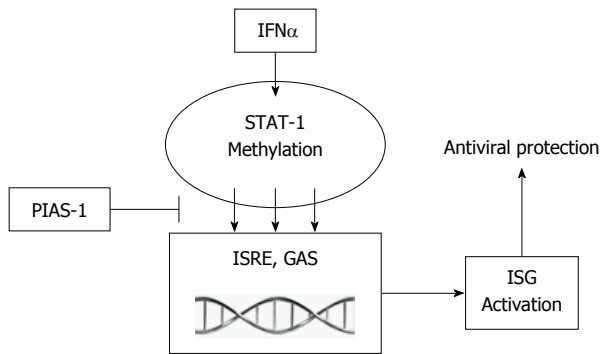


Figure 1 Acetaldehyde suppresses interferon- α signaling in hepatitis C virus -infected liver cells by impairing signal transducers and activators of transcription 1 methylation. The most downstream event in interferon (IFN) α signaling is the attachment of methylated STAT1 to DNA, interferon stimulated response element (ISRE) and gamma-interferon activated site (GAS), for activation of anti-viral interferon-stimulated genes (ISGs). Acetaldehyde suppresses STAT1 methylation, which facilitates increased STAT1 interaction with protein inhibitor of activated STAT 1 (PIAS1, a negative regulator of IFN signaling) preventing STAT1 binding to DNA. This ultimately results in reduced ISG activation and decreased induction of anti-viral proteins.

impact on the function of various metabolic pathways exacerbating alcohol-induced hepatic injury. Many lines of evidence, from animals to humans, have shown that dietary factors, including dietary fat, along with heavy alcohol consumption, play critical roles in the ALD pathogenesis. Indeed, the relative beneficial effects of dietary saturated fat (SF) and damaging effects of dietary unsaturated fat [USF, primarily corn oil/linoleic acid (LA) enriched] on alcohol-induced liver injury have been well documented in experimental animal models of ALD^[66-72]. A number of mechanisms have been proposed for the opposing effects of dietary USF vs SF in ALD, including (1) induction of lipid peroxidation and oxidative stress^[70,73,74]; (2) altered gut microbiota, impaired intestinal barrier integrity, endotoxemia, and associated increase in liver pro-inflammatory cytokine production^[66,67,72]; (3) modulation of hepatic lipid metabolism *via* SIRT1-SREBP-1-histone H3 axis^[75]; and (4) modulation of hepatocyte nuclear factor-4 α expression, a master transcription factor in the regulation of lipid metabolism^[76]. A new concept has recently emerged that the bioactive oxidized LA metabolites (OXLAMs), which are formed enzymatically from LA primarily *via* the actions of 12/15-lipoxygenase (12/15-LOX), or non-enzymatically *via* free radical-mediated oxidation in response to oxidative stress, might contribute to ALD pathogenesis. It has been demonstrated that plasma OXLAMs, specifically 9- and 13-hydroxy-octadecadienoic acids (9- and 13-HODEs), were elevated in patients with alcoholic cirrhosis in parallel with the increase in lipoxygenases (15-LOX-1 and 15-LOX-2 mRNA) in the liver samples. The plasma levels of HODEs in patients with ALD were significantly higher than in healthy subjects as well as in NAFLD patients^[77]. Further, increased levels of 9- and 13-HODEs were observed in experimental animal models of ALD^[78,79] in parallel with the hepatic

steatosis, oxidative stress, and inflammation and hepatocyte damage. It has been reported that 9- and 13-HODEs are natural endogenous ligands for the Transient Receptor Potential Vanilloid 1 (TRPV1)^[80,81]. Our recent study demonstrated that chronic-binge ethanol-mediated increases in circulating OXLAMs and TRPV1 levels in mice were associated with hepatic steatosis, inflammation and injury^[78]. Genetic depletion of TRPV1 did not blunt hepatic steatosis caused by ethanol, but prevented hepatic injury. TRPV1 deficiency protected from hepatocyte death and prevented the increase in pro-inflammatory cytokine and chemokine expression, including TNF- α , interleukin-6, macrophage inflammatory protein-2 and monocyte chemoattractant protein-1. Moreover, TRPV1 depletion markedly blunted ethanol-mediated induction of plasminogen activator inhibitor-1, an important mediator of alcohol-induced hepatic inflammation, *via* fibrin accumulation^[78]. Exposure of HepG2 cells to 9- and 13-HODEs resulted in activation of TRPV1 signal transduction with the increased intracellular Ca²⁺ levels, suggesting that OXLAM/TRPV1/Ca²⁺ signaling may be a relevant pathway contributing to ALD pathogenesis.

Alcohol consumption increases hepatic oxidative stress with the production of reactive oxygen species. One of the major sources of *in vivo* protein modification during oxidative stress is thought to be oxidative products of polyunsaturated fatty acids (PUFAs). LA is the most abundant PUFA in mammalian tissue. Non-enzymatic oxidative degradation of PUFAs, including LA, generate a variety of lipid peroxidation products (LPOs, *e.g.*, MDA, HNE, acrolein, various epoxyketo-octadecenoic acid isomers)^[11]. Enhanced amounts of peroxidized phospholipids and their truncation products in the circulation have previously been observed in rats with alcohol-induced liver disease^[79]. Some LPOs can modify DNA, peptides and proteins leading to formation of advanced lipoxidation end-products (ALEs). These modifications can potentially cause functional damage to proteins. Numerous LPOs and ALEs exert diverse biologic activities (*e.g.*, damaging and pro-inflammatory effects in some cases) through different as yet not well-defined mechanisms. The role and the significance of oxidized lipids, both dietary and *in vivo*-produced, as well as possible mechanisms underlying their beneficial or deleterious effects in liver pathology remain to be determined.

CONCLUSION

In summary, chronic ethanol consumption dysregulates post-translational modifications of numerous important proteins that regulate many cellular processes. Not surprisingly, there is considerable overlap in the pathways that are targeted. Understanding how each individual modification affects specific protein function and thereby, alters metabolic pathways will

be of critical importance to deciphering the impact of the aforementioned modifications to alcohol-induced steatosis and hepatocellular damage. It could also provide an insight into disease pathogenesis and progression, and may help to identify additional useful targets of drug action. In addition, the activation of enzymatic and/or non-enzymatic degradation of polyunsaturated fatty acids resulting in the formation of numerous bioactive lipid compounds underlies the deleterious effects of certain dietary fat intake in promoting indices of alcoholic liver damage.

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Pleiotropic effects of statins in the diseases of the liver

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CoA reductase. They are usually prescribed as a lipid lowering medication. However, there is accumulating evidence that statins have multiple secondary effects both related and unrelated to their lipid-lowering effect. This narrative review of the literature aims to provide the reader with information from clinical studies related to the effect of statin and statins' potential use in patients with liver diseases. In patients with advanced liver disease due to any etiology, statins exhibit an antifibrotic effect possibly through the prevention of hepatic sinusoidal microthrombosis. Two randomized controlled trials confirmed that statins decrease hepatic vein pressure gradient in patients with portal hypertension and improve the survival of patients after variceal bleeding. Lower rates of infections were observed in patients with cirrhosis who received statin treatment. Statins decrease the risk of hepatocellular carcinoma (HCC) in patients with advanced liver disease in general but particularly in patients with chronic hepatitis B and C. Statins in patients with chronic hepatitis C likely increase the virological response to the treatment with pegylated interferon and ribavirin and have the potential to decrease the rate of fibrosis. Finally, data from randomized controlled trials also confirmed that the addition of statin prolongs the survival of patients with advanced HCC even more than sorafenib. Statins are a very promising group of drugs especially in patients with liver disease, where therapeutic options can often be limited. Some indications, such as the prevention of re-bleeding from esophageal varices and the palliative treatment of HCC have been proven through randomized controlled trials, while additional indications still need to be confirmed through prospective studies.

Key words: Statins; Hepatitis; Cirrhosis; Esophageal varices; Hepatocellular carcinoma

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Abstract

Statins are a class of molecules that inhibit HMG

Core tip: The greatest benefit of statins seems to be in patients with advanced liver disease. Observational

studies suggest that statins have an antifibrotic effect possibly through the prevention of hepatic sinusoidal microthrombosis, reduce the rate of infections and decrease the risk of hepatocellular carcinoma in all cirrhotics, but particularly in patients with chronic hepatitis B and C. Data from randomized controlled trials confirmed that statins decrease hepatic vein pressure gradient, prevent re-bleeding, and improve the survival of patients after variceal bleeding. Statins also seem to prolong the survival of patients with advanced hepatocellular carcinoma even more than those treated with Sorafenib, which is the current standard of care for these patients.

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INTRODUCTION

Statins are an inhomogeneous group of molecules that inhibit the activity of hydroxymethylglutaryl-coenzyme A reductase (HMG CoA reductase), a key enzyme in the synthesis of cholesterol. Statins were discovered as a byproduct in the search for new antimicrobial agents. The first statin (mevastatin) was the product of Penicillium citrinum, but its clinical use was abandoned due to hepatotoxicity^[1]. Lovastatin was the first clinically successful statin to be used effectively^[2]. Scandinavian simvastatin survival study (4S) confirmed that statins reduce cardiovascular as well as general mortality in patients with atherosclerosis^[3].

Individual molecules from the statin group differ in several important attributes. Lovastatin, simvastatin, fluvastatin and atorvastatin are lipophilic, whereas pravastatin and rosuvastatin are hydrophilic. Pravastatin is metabolized in the liver by sulfation, while lovastatin, simvastatin and atorvastatin are metabolized by cytochrome P-450 3A4. Fluvastatin and rosuvastatin are metabolized partially by cytochrome P-450 2C9^[4].

Besides lipid lowering properties statins also exhibit multiple pleiotropic effects, which could be detrimental (*i.e.*, adverse effects) or beneficial. It is unknown whether the pleiotropic effects are directly related to the primary effect of the drug. Statins exhibit various antiatherogenic effects, such as the improvement of endothelial function, antioxidative, antiproliferative and antiinflammatory properties as well as neoangiogenesis^[4,5]. Additionally, statins reduce the risk of sudden cardiac death, deep vein thrombosis and fibrosclerotic aortic stenosis, while treatment with statins could influence the regression of left ventricular hypertrophy^[4]. Statins also exhibit multiple non-

cardiovascular effects. For example, a cross-sectional analysis of three hospital databases showed that patients using statin had a 60% lower prevalence of Alzheimer's disease^[6]. Besides Alzheimer's, statins also reduce the risk of other types of dementia^[7] and have a lower prevalence of vitiligo, osteoporosis, rheumatoid arthritis and sclerosis multiplex^[4]. Increased risk of type 2 diabetes mellitus could be counted among the negative pleiotropic (adverse) effects of statin therapy^[8].

Non-alcoholic steatosis (NAFLD) of the liver is considered a hepatic manifestation of metabolic syndrome^[9]. While the exact causality is unknown, the significant increase of both subcutaneous and visceral fat along with dyslipoproteinemia in the majority of these patients is closely associated with the accumulation of fat in the liver. In some patients the hepatic fat stimulates an inflammatory response that causes non-alcoholic steatohepatitis that in turn could progress to liver cirrhosis. Besides liver related morbidity and mortality, the presence of NAFLD is a significant and independent risk factor for cardiovascular events^[10]. Statins are prescribed in these patients to positively influence lipoprotein metabolism. Moreover, increasing evidence suggests that statins improve all aspects of NAFLD. Statins decrease the elevated plasmatic activity of liver enzymes^[11], while statins in monotherapy and in combination with antioxidants decrease hepatic fat accumulation^[12,13]. Prolonged administration of statins could also reduce liver fibrosis^[12]. Interestingly one study showed that statin therapy longer than two years in obese patients reduces the prevalence of liver steatosis^[14]. Statin treatment also reduces the risk of cardiovascular mortality, and the risk reduction is significantly greater in patients with elevated liver enzymes^[11].

Another well-established indication for statins are cholestatic liver diseases, particularly primary biliary cholangitis, which is commonly associated with elevated total and LDL-cholesterol levels, and statins could partially reverse this negative effect^[15].

STATINS AND CHRONIC VIRAL HEPATITIS B/C

Chronic viral hepatitis B and C could progress to liver cirrhosis, which increases the risk of developing hepatocellular cancer (HCC). Chronic viral hepatitis, particularly hepatitis B, could lead to hepatocellular cancer even without cirrhosis^[16,17].

The aim of the treatment of chronic hepatitis C is the elimination of the virus. An undetectable virus 24 wk after the end of treatment is termed "sustained viral response (SVR)". Interferon based therapy was the standard of care of chronic hepatitis C patients before direct-acting antivirals became available. The

rate of SVR for interferon based therapy depends on multiple factors (*IL28B* gene polymorphisms, pre-treatment hepatitis C virus (HCV) viral load, HCV reduction dynamics, the degree of fibrosis, etc.)^[18].

Statins and HCV RNA without concomitant antiviral treatment

Multiple authors have reported the effect of statin treatment on HCV viral load. An *in-vitro* study conducted by Ikeda *et al.*^[19] showed that fluvastatin, lovastatin, simvastatin and atorvastatin prevent the replication of HCV RNA, and that this effect is significantly stronger in fluvastatin compared to other statins. *In vivo* studies showed varied results. Forde *et al.*^[20] compared three groups of patients with chronic hepatitis C. Group A consisted of patients with dyslipidemia on statin treatment (without specification) for at least 60 d prior to the HCV RNA quantification, group B included dyslipidemic patients without statin, and group C included patients without dyslipidemia and not on statin treatment. The authors did not report significant differences in HCV RNA levels among these three groups of patients. Fluvastatin dosed 80 mg daily led to the reduction of HCV RNA in 50% of patients, with the highest weekly reduction by 1.75 decadic logarithm. The reduction of HCV RNA occurred in the first four weeks of treatment in 82% patients with viral response. However, after the reduction of the dose the HCV RNA increased in 22% of responders in the following 2-5 wk^[21]. Another observational study from Romania showed a significant decrease of HCV RNA after treatment with either 40 mg of fluvastatin or 20 mg of lovastatin (mean levels of HCV RNA before treatment 2376074 ± 3427596 IU/mL, and 1321136 ± 1343570 IU/mL after treatment, $P = 0.001$). The administration of both statins was associated with significant reduction of proinflammatory signaling by IL6 and TNF- α , while the fluvastatin group also had lower IL-8 levels^[22]. On the other hand, a study by Sheridan *et al.*^[23] did not find significant differences in HCV RNA levels between patients treated with 40-80 mg of fluvastatin (+/- ω -3-polyunsaturated fatty acids) and controls after 12 wk of treatment. The main limitation of this study is, that it included 35% of patients that had already been diagnosed with cirrhosis and 45% that were non-responders to PEG IFN treatment. Fluvastatin treatment also had a surprisingly negative effect in HCV/HIV coinfecting patients, where it led to a mild increase of HCV RNA (HCV RNA before treatment 5.63 ± 0.5 log₁₀ IU/mL vs 5.84 ± 0.6 log₁₀ IU/mL after treatment, $P = 0.001$), compared to no change in HCV RNA in the control group^[24].

The effect of other statins on HCV RNA has not been proven in any studies. Simvastatin treatment for three months did not affect HCV RNA levels significantly^[25] and neither did the combination of simvastatin with sertraline^[26]. Twelve weeks treatment with rosuvastatin

titrated to 40 mg daily led to the decrease of HCV RNA higher than one decadic logarithm only in one out of eleven patients^[27]. A meta-analysis showed a relatively small but significant decrease of HCV RNA (0.2 decadic logarithm decrease, 95%CI: 0.09-0.31, $P < 0.001$) in patients treated with fluvastatin, but lovastatin, simvastatin, atorvastatin and rosuvastatin had no effect on HCV RNA levels^[28]. These results suggest that standard statin therapy does not have a significant effect on the dynamics of HCV RNA viral load, with the possible exception of fluvastatin.

Despite the dubious effects of statins on HCV viral load, there is a distinctive antifibrotic effect of this treatment in HCV infected patients. The data comes from a large observational study from Taiwan, performed in 1997-2010 included 226856 patients with chronic hepatitis C. Cirrhosis was present in 34273 patients. The incidence of cirrhosis during the follow-up was significantly higher in patients not taking statins (1311.2 vs 445.5 cases per 100000 person-years) Hazard ratios were 0.33 (95%CI: 0.31-0.36), 0.24 (95%CI: 0.22-0.25), and 0.13 (95%CI: 0.12-0.15) when statin users were compared with non-statin users with cumulative defined daily doses (cDDD) of 28-83, 84-365, and greater than 365 respectively^[29].

Statins and HCV RNA with concomitant antiviral treatment

The possibility of improving the treatment efficacy of standard antiviral treatment with the addition of statins has been evaluated with interferon based treatment, which was the standard of care before the development of direct acting antivirals. The efficacy of pegylated interferon with ribavirin was about 50%^[30,31]. The addition of a statin effectively enhanced the antiviral effect of this treatment, particularly fluvastatin, exhibiting synergistic inhibitory effect on HCV RNA replication^[19]. Several studies have explored this synergy in studies *In vivo*. Japanese authors reported the SVR rate in patients treated with PEG IFN and ribavirin with the addition of 20 mg fluvastatin to be as high as 67%, however it is important to note that this observational study did not have any control group^[32]. Another study explored the addition of 20 mg fluvastatin to PEG IFN + ribavirin treatment (46 patients) and compared them to a control group (48 patients). The duration of treatment was 48 wk in patients with complete early viral response (cEVR) and 72 wk in patients without cEVR, but with HCV RNA negativity in 13-36 wk of treatment. There was no difference in cEVR between the statin and control group (50% vs 54.2%), but patients with cEVR achieved SVR in the fluvastatin group more frequently than the control group (91.3% vs 65.4%, $P = 0.042$). Furthermore, patients in the control group relapsed significantly more often than in the fluvastatin group (39.4% vs 14.7% respectively, $P = 0.027$)^[33]. There is also anecdotal evidence that the addition of

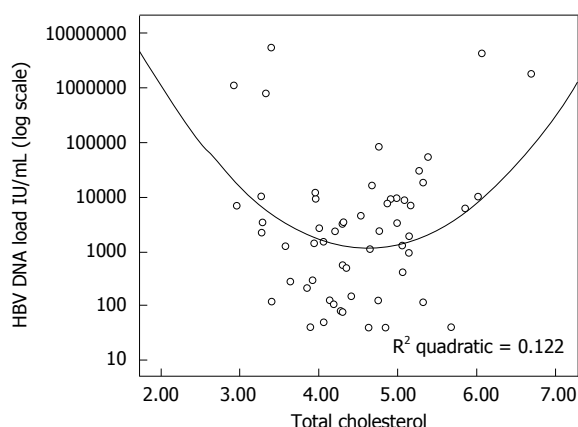


Figure 1 Association between total cholesterol and hepatitis B virus DNA load^[43]. With permission from Elsevier. HBV: Hepatitis B virus.

pitavastatin and eicosapentaenoic acid to the PEG IFN + ribavirin treatment could increase the rate of achieving SVR^[34]. These results were confirmed in a meta-analysis of five different studies that included fluvastatin, simvastatin, rosuvastatin and pitavastatin. The addition of statin doubled the chance of SVR (OR = 2.02, 95%CI: 1.38-2.94) as well as rapid and early viral responses^[35].

The addition of statin to the PEG IFN + ribavirin treatment could influence not only the SVR, but also the risk of complications, such as progressive fibrosis or HCC. The HALT-C study included non-responders to the previous interferon based treatment with advanced fibrosis (Ishak score ≥ 3). Patients that did not achieve virological response (HCV RNA negativity in week 20) were treated with PEG IFN + ribavirin for the next 3.5 years. Patients who were concomitantly treated with statin displayed a decrease of liver fibrosis (-0.34 ± 0.94 points) compared to non-statin users, where fibrosis progressed (0.42 ± 1.42), $P = 0.006$. Overall, fibrosis progression was found in 10% of statin users and 29% of non-users (adjusted HR = 0.31, 95%CI: 0.10-0.97). Statin treatment did not significantly influence the histology activity index or the plasmatic activity of ALT^[36]. Similar data was reported from a registry-based study (ERCHIVES Registry) of 9135 HCV infected veterans treated with interferon based therapy in the years 2001-2014. Liver cirrhosis occurred in 1649 patients and HCC in 239 patients. The risk of cirrhosis in statin users was 44% lower (adjusted HR = 0.6, 95%CI: 0.53-0.68) and the risk of HCC was lower by 49% (adjusted HR = 0.51, 95%CI: 0.36-0.72) compared to non-users. The strongest antifibrotic effect was attributed to atorvastatin and fluvastatin^[37].

The addition of statin to the interferon-based therapy has the potential to decrease the degree of fibrosis and the risk of HCC; however, patients with chronic hepatitis C receive statins less frequently compared to patients without HCV infection^[38]. The introduction of direct acting antivirals, with SVR rates up to 100% also

in cirrhotics and nonresponders to previous IFN based treatment, limits the benefit of statin treatment in HCV infected patients^[39-41]. However, statins may potentially play a role in other aspects of chronic HCV infection^[42].

There is no relevant information about the statin influence on hepatitis B virus both in terms of hepatitis B virus (HBV) DNA dynamics or fibrogenesis. However, in an earlier study we reported that cholesterol has a significant quadratic relationship with HBV DNA. Thus, patients with cholesterol levels above and below the normal range had higher levels of HBV DNA (Figure 1)^[43]. It has been well documented that HBV DNA level is the strongest predictor of fibrosis progression. According to Iloeje *et al.*^[44] "the cumulative incidence of cirrhosis is 4.5% in patients with HBV DNA < 300 copies/mL compared to 36.2% in patients with HBV DNA $\geq 10^6$ copies/mL ($P < 0.001$)". However, it is unclear if statin treatment in hypercholesterolemic patients would in any way influence HBV DNA levels. There is some in vitro data that simvastatin might increase the antiviral activity of nucleot(s)ide analogues^[45], but it is unlikely that this information will have any clinical meaning, because of the high efficacy of currently available tenofovir and entecavir.

STATINS AND FIBROGENESIS

Statins in general positively influence endothelial dysfunction and this effect is also present in intrahepatic sinusoids. They show an anti-inflammatory effect in the inflammatory response caused by endotoxin, angiotensin II or hypovolemia, and the diminished activation of hepatic stellate cells (HSC)^[46]. Statins inhibit non-canonical hedgehog signaling and cirrhotic portal hypertension^[47], resulting in a protective effect in ischemic hepatitis^[48] and have protective effects against the thrombosis of hepatic sinusoids and portal vein^[49].

Parenchymal extinction theory of fibrogenesis proposes the microthrombosis of liver sinusoids as the driving force of inflammation and fibrosis. This is supported by the frequent finding of factor V Leiden mutations, protein C deficiency and increased factor VIII expression in cirrhotics^[50]. Thrombin, generated as the result of coagulation cascade activation, might activate HSC through protease activated receptors 1 and 4^[51]. The administration of statin in these circumstances increases protein C activity^[52] and decreases thrombin generation in plasma^[53]. One of the key prothrombotic factors in liver cirrhosis is von Willebrand factor antigen (vWF:Ag). This is released by endothelial cells and megacaryocytes and promotes the endothelial adhesion of thrombocytes, the transport and binding of factor VIII and thrombus formation. The level of vWF:Ag directly correlates with the degree of liver fibrosis (Figure 2). Maieron *et al.*^[54] developed a novel scoring system, that included vWF:Ag divided by thrombocytes (VITRO) for the prediction of liver cirrhosis with AUC 0.893 compared

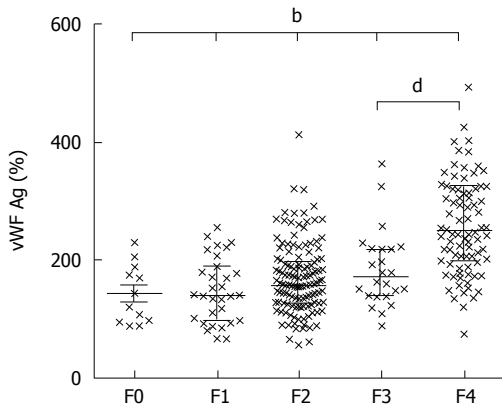


Figure 2 Dotplots for vWF:Ag according to fibrosis stage showing mean values and IQR. ^b $P < 0.001$ for all fibrosis stages, F3 vs F4 ^d $P < 0.0001$ ^[54]. With permission from John Wiley and sons.

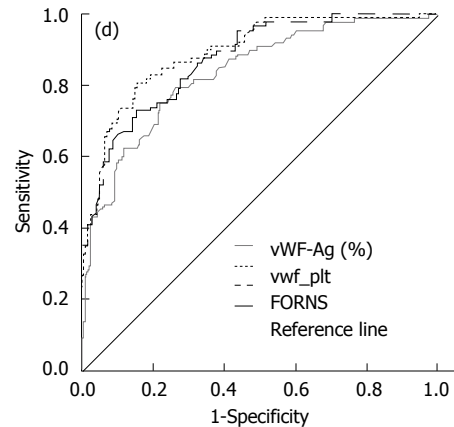


Figure 3 Receiver operating characteristics curves for vWF:Ag, VITRO score and FORNS in the diagnosis of cirrhosis (F4). AUC vWF:Ag = 0.835, VITRO score = 0.893 and FORNS = 0.874 ($P = NS$)^[54]. With permission from John Wiley and sons.

to Forns score AUC 0.874, $P = NS$ (Figure 3). The VITRO score is also more accurate for the noninvasive diagnosis clinically significant portal hypertension than the ELF or APRI score^[55]. Simvastatin and pravastatin significantly decrease vWF:Ag levels [SMD: -0.54, 95%CI: -0.87-(-0.21), $P = 0.001$], in contrast to fluvastatin, atorvastatin and rosuvastatin. The greatest decrease of vWF:Ag level was observed after 12 wk of statin administration^[56]. Statins in animal models upregulate Kruppel-like factor 2 (KLF2) signaling pathways that leads to the decrease of circulating vWF:Ag, decreased activation of HSC, and the regression of liver fibrosis^[46,57,58].

Increasing evidence from interventional studies provides support for the microthrombotic theory of fibrosis. The Italian authors evaluated 48 wk of enoxaparin treatment in cirrhotic patients (Child Pugh 7-10 points) with parent portal vein. Enoxaparin decreased not only the incidence of portal vein thrombosis, but also the incidence of decompensation and mortality^[49]. It is unclear if statin treatment alone could decrease the incidence of portal vein thrombosis in cirrhotics; however, in patients with diagnosed malignancy, this treatment significantly decreases the cumulative incidence of deep vein thrombosis. Six months after the start of the statin the incidence of deep vein thrombosis was only sporadic^[59]. This data indicates the need to further study statins in compensated and decompensated cirrhotics aimed at the prevention of portal vein thrombosis.

STATINS AND THE CLINICAL COURSE OF CIRRHOSIS

Two retrospective observational studies evaluated the effect of statins on clinical outcomes of cirrhotic patients. The first study included 81 cirrhotics on statin treatment and 162 controls. The median follow-up was 36 mo in the statin group and 30 mo in the control group. There was no difference in etiology,

age, Child-Pugh, MELD, HCC prevalence, beta blockers use, esophageal varices or selected biochemical parameters at inclusion. Patients in the statin group had a significantly higher prevalence of coronary heart disease and a lower prevalence of diabetes mellitus. The mean survival time of patients in the statin group was 10.8 years compared to 6.3 years in the control group ($P = 0.06$). The mean survival time of Child Pugh A patients was 14.4 years in the statin group and 7 years in the control group ($P = 0.01$). The adjusted hazard ratio for overall mortality was 0.53, $P = 0.01$ in statin users. The authors also reported lower risk of cirrhosis decompensation in statin users^[60]. Another study performed by Mohanty *et al*^[61] was registry-based and included patients with cirrhosis caused by hepatitis C infection between 1996-2009. The study cohort included 40 512 patients, 98% of which were male with an average age of 56 years, 2802 patients were using statins. The authors compared the propensity matched cohorts of statin users and non-users and found that patients using statin had a lower risk of decompensation (HR = 0.55, 95%CI: 0.39-0.77) and death (HR = 0.56, 95%CI: 0.46-0.69). These observational studies provide a solid foundation to consider a randomized controlled trial with statin in liver cirrhosis, despite the already decreased level of cholesterol in cirrhotics that correlates with the prognosis^[62].

STATINS AND PORTAL HYPERTENSION

Changes in intrahepatic microcirculation, increased intrahepatic vascular resistance and splanchnic vasodilation are the main factors leading to portal hypertension^[63]. Nitric oxide (NO) is the main modulator of the vascular tonus both in the liver and in the splanchnic region. The physiological production of NO is associated with anti-fibrotic, anti-inflammatory and anti-thrombotic effects. Decreased NO production in the sinusoidal endothelial cells has a proinflammatory and

profibrotic effect in the liver^[48,64]. Simvastatin increases NO production in hepatosplanchnic region, decreases vascular resistance, and ameliorates the postprandial increase of portal pressure in cirrhotic patients without a substantial effect on systemic circulation^[65]. Abraldes *et al*^[66] performed a randomized controlled trial in patients with portal hypertension that evaluated the efficacy of 20 mg simvastatin, later titrated to 40 mg on the hepatic vein pressure gradient (HVPG). The decrease of HVPG was greater in the statin group ($8.3\% \pm 12.2\%$ vs $1.6\% \pm 12.3\%$, $P = 0.041$). Statin treatment led to the decrease of HVPG both in patients treated (-11% , $P = 0.033$) and not treated (-5.9% , $P = 0.013$) with beta-blocker. Statin treatment did not affect systemic circulation and the incidence of adverse effects was the same in the treatment and control group. Another prospective study by Pollo-Flores *et al*^[67] included 34 patients with portal hypertension. Fourteen patients received 40 mg of simvastatin and 20 patients received placebo for 3 mo. Three patients in the statin group were excluded because of a contrast medium reaction and newly diagnosed HCC, while seven patients were excluded from the control group. In the per-protocol analysis the authors reported the decrease of HVPG in the statin group compared to no change in the control group (2 ± 2.2 Torr vs 0 ± 1.1 Torr, $P = 0.02$). Primary endpoint (the decrease HVPG of at least 20% from the baseline or under 12 mmHg) was achieved in 55% of patients in the statin group and 0% of patients in the control group ($P = 0.036$). Clinical outcomes related to portal hypertension, particularly variceal re-bleeding, have been evaluated in the BLEPS study which was a multicenter double-blind randomized controlled trial. It included 69 patients in the active group that received 20 mg of simvastatin titrated to 40 mg after 15 d and 78 patients in the control group. Patients were followed up for 24 mo. The primary endpoint was re-bleeding or death. Nine percent of patients in the statin group and 22% of patients in the control group died during the study (HR = 0.39, 95%CI: 0.15-0.99, $P = 0.030$). Simvastatin treatment reduced the relative risk of death compared to the placebo by 61%. The rate of re-bleeding did not differ significantly between the two groups. Two patients from the statin group developed rhabdomyolysis during the statin treatment^[68]. As practically all of the studies used simvastatin it is not clear if this effect is a class effect of all statins or is limited to simvastatin.

STATINS AND INFECTIONS IN CIRRHOSIS

Infections are common in cirrhotic patients and increase mortality by approximately four-fold. Thirty percent of patients die in the first month after infection diagnosis and another 30% in the following year^[69]. Motzkus-Feagans *et al*^[70] evaluated the effect of statin treatment on the incidence of infections. The study

included 19379 patients with compensated cirrhosis from United States Veterans Health Administration database, with a mean follow-up of 1194 (365-3103) d. 2468 patients were receiving statin, the most common was simvastatin. Infection was diagnosed in 12.4% of patients during follow-up, with a mean time to infection of 608 d. The most common infections were pneumonia and skin infections. Statin treatment was associated with reduced infection rate and mortality rate in the whole cohort (aHR = 0.42, 95%CI: 0.36-0.48), as well as in the propensity score matched sample that included 503 statin users and 1760 statin non-users (aHR = 0.67, 95%CI: 0.47-0.95)^[70]. The question remains if statins improve the outcome of patients with severe infection or sepsis. Although no data exists about this particular issue in cirrhotic patients, there are many studies about this topic in the general population. Meta-analysis showed that patients with severe infections or sepsis, who were given statin had lower mortality for sepsis [aOR = 0.40 (95%CI: 0.23-0.57)], pneumonia [aOR = 0.33 (95%CI: 0.09-0.75)] and mixed infection-related mortality [aOR = 0.50 (95%CI: 0.18-0.83)] compared to statin non-users^[71]. These findings, however, were not confirmed in the randomized placebo-controlled trial with 40 mg of atorvastatin. Although statin treatment reduced the conversion rate from sepsis to severe sepsis (4% vs 24%, $P = 0.007$), no significant differences were found in mortality, length of hospital stay, or the number of re-hospitalizations^[72]. Therefore, more studies are needed to evaluate the clinical benefit of statins in cirrhotics with infections or sepsis.

STATINS AND HCC

Statin treatment does not generally affect the incidence of cancer or cancer related mortality^[73]. Hepatocellular carcinoma (HCC) occurs mostly in cirrhotic liver, with less than 20% of HCC occurring in non-cirrhotic liver^[74]. Therefore, statin therapy may indirectly influence the risk of HCC with its anti-fibrotic effect. Accumulated evidence from mostly observational studies suggest, that statins could also decrease the incidence of HCC by direct chemopreventive effect. The carcinogenesis of HCC along with potential targets for prophylaxis or treatment is depicted on Figure 4. Multiple target sites of statins include the inhibition of post-translational prenylation of Ras/Raf proteins, inhibition of the proteasome pathway activation, limitation of the cyclin-dependent kinase inhibitors p21 and p27 degradation, and the blocking of Myc phosphorylation and activation, suppressing tumor initiation and growth^[75].

Multiple clinical studies evaluated the effect of statin treatment on the incidence of HCC. Singh *et al*^[76] included 10 of the studies in the meta-analysis published in 2013. Seven studies were observational (3 case-control and 4 cohort studies) and three were RCTs, six studies included Western population and four

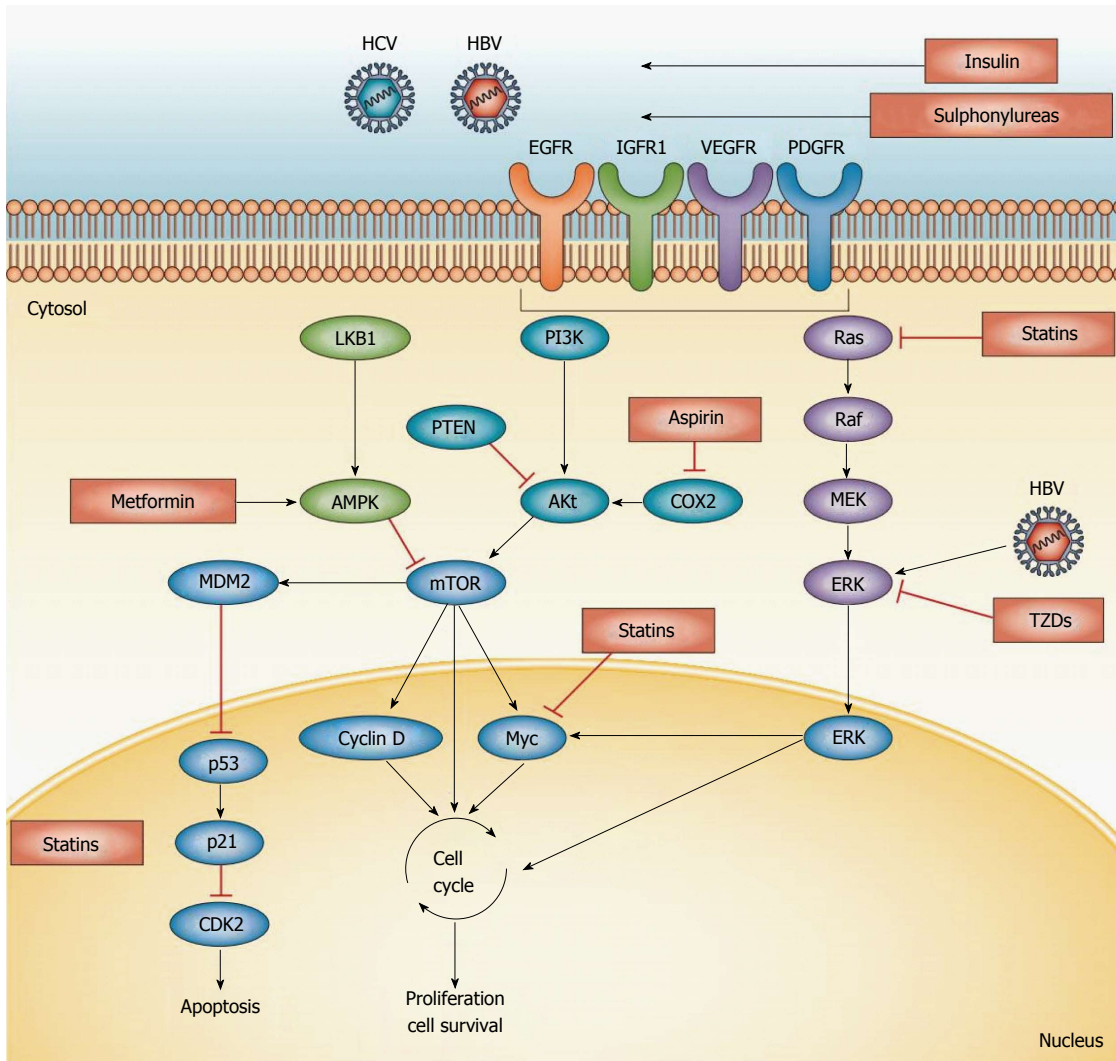
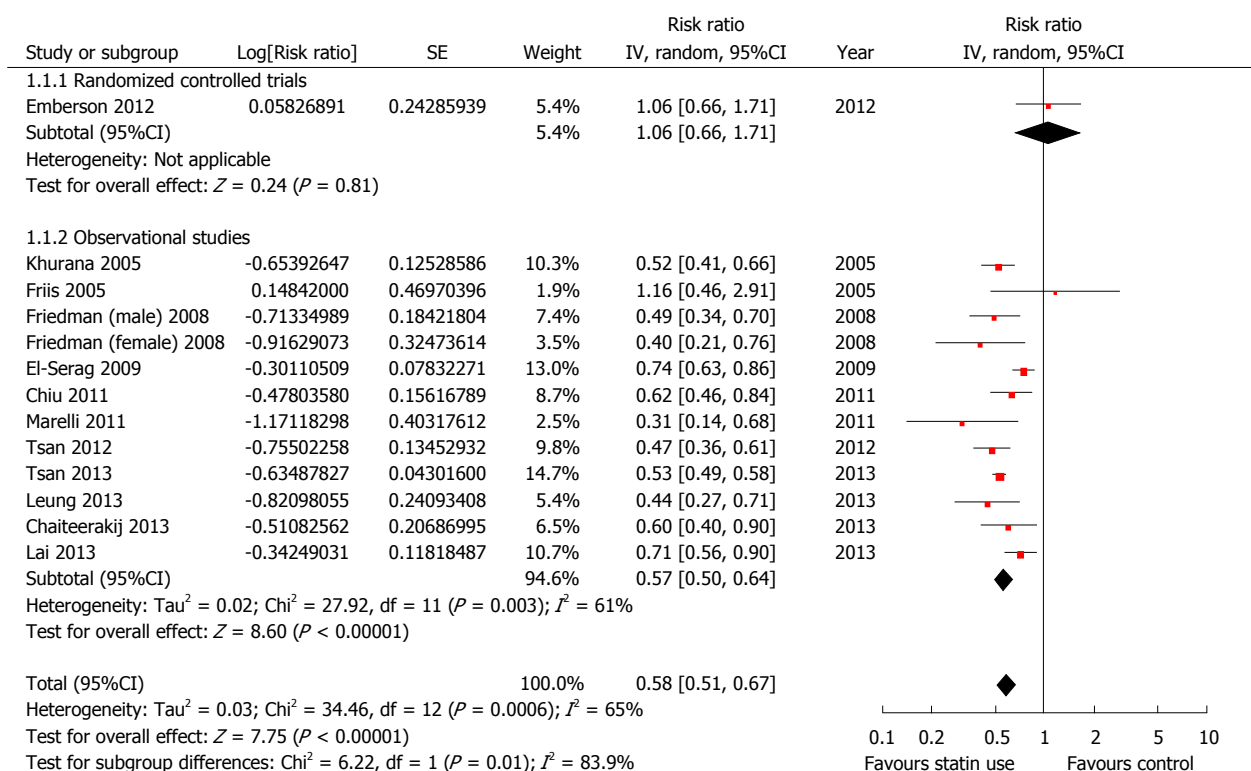


Figure 4 Pathogenesis of hepatocellular carcinoma and targets for chemopreventive agents. Tyrosine kinase associated receptor pathways induce MAPK and PI3K-Akt kinase pathways in > 50% of HCCs. The resulting disruption of the mTOR pathway is seen in 40%-50% of cases of HCC, leading to inactivation of tumour suppressors such as PTEN. Statins block post-translational prenylation of Ras/Raf proteins, inhibit the activation of the proteasome pathway, limiting the degradation of the cyclin-dependent kinase inhibitors p21 and p27, and block Myc phosphorylation. Metformin activates AMPK, which inhibits the mTOR pathway. Thiazolidinediones inhibit the ubiquitin-proteasome system and extracellular signal-regulated kinase pathway. Insulin and sulphonylureas might promote hepatocarcinogenesis by increasing IGFR1 activity, enhancing growth-factor-dependent cell proliferation. AMPK: Adenosine monophosphate-activated protein kinase; HCC: Hepatocellular carcinoma; IGFR1: Insulin-like growth factor receptor 1; IR: Insulin receptor; MAPK: Ras mitogenactivated protein kinase; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol 3-kinase; PPAR- γ : Peroxisome proliferator activated receptor γ ^[75]. With permission from Nature publishing group.

studies Asian population^[73,77-85]. A total of 1459417 patients were included. The chemopreventive effect of statin administration was reported in half of the studies. Overall, statin administration was associated with lower risk of HCC (aOR = 0.63, 95%CI: 0.52-0.76). The risk of HCC was lower in statin users in both the Western (aOR = 0.67, 95%CI: 0.53-0.85) and Asian population (aOR = 0.52, 95%CI: 0.42-0.64). These findings were confirmed in an updated meta-analysis by Shi *et al*^[86] that included 12 studies (6 case-control studies, 5 cohort studies and 1 randomised controlled study)^[73,77-82,85,87-90]. The relative risk of HCC in statin users was 0.58, (95%CI: 0.51-0.6), Figure 5. A smaller meta-analysis by Zhou *et al*^[91] that included five observational studies^[78,85,87,92,93], also showed a significant risk reduction of HCC in statin users. Odds

ratios were 0.63, 95%CI: 0.45-0.89 for atorvastatin and OR = 0.58, 95%CI: 0.40-0.85 for fluvastatin. The chemopreventive effect of statins was also described in patients with chronic hepatitis C without cirrhosis. A registry-based study from Taiwan included 35023 statin users and 225841 non-statin users. The authors reported a significant dose response relationship between statin use and the risk of HCC with an aHR of 0.66, 95%CI: 0.59-0.74, aHR = 0.47, 95%CI: 0.40-0.56 and aHR = 0.33, 95%CI: 0.25-0.42) in groups with cDD of 28-89, 90-180 and more than 180 respectively^[90].

The incidence of HCC in patients with chronic hepatitis B partially depends on the viral load. Levels of HBV DNA ≥ 10000 copies/mL are a significant predictor of HCC independent of ALT levels, HBeAg

Figure 5 Overall meta-analysis of statin use and liver cancer risk^[88].

or the presence of liver cirrhosis^[94]. Statin use significantly decreased the risk of HCC in patients with hepatitis B in a registry-based observation from Taiwan with dose-dependent relationship. Adjusted hazard ratios were 0.66, 95%CI: 0.44-0.99, 0.41, 95%CI: 0.27-0.61 and 0.34, 95%CI: 0.18-0.67 for cDDD of 28-90, 91-365 and greater than 365 respectively^[85]. Another study from Hong Kong also reported that statin use was associated with 32% risk reduction of HCC development [weighted sub-hazard ratio (SHR) = 0.68; 95%CI: 0.48-0.97], however statins did not reduce the risk of mortality (weighted HR = 0.92, 95%CI: 0.76-1.11). The addition of statin to the standard nucleot(s)ide analogue treatment reduced the risk of HCC by 59% (weighted SHR 0.41, 95%CI: 0.19-0.89) compared to patients with only nucleot(s)ide analogue treatment^[95]. This corresponds with the presumed synergy between nucleot(s)ide analogue and statins for HCC risk reduction^[45].

It has been shown that statins do not influence the incidence of cancer in the general population^[73]. Interestingly, the risk reduction seems to be significant in patients with chronic hepatitis B. An observational study by Chen *et al*^[96] included 71847 patients with chronic hepatitis B. Statin users from this study had significantly lower risk of not only liver cancer but also all malignancies in general. The concomitant use of statin and metformin reduced the risk of malignancies even further in patients with chronic hepatitis B (Table 1).

Statins have also been tried as a concomitant therapy in patients with confirmed HCC. Two ran-

domized controlled trials evaluated the role of statins in the treatment of advanced hepatocellular carcinoma. Japanese authors randomized 83 patients with non-resectable HCC undergoing transarterial chemoembolisation into 40 mg pravastatin and control group. The mean survival rate was significantly longer in the statin group (18 mo vs 9 mo)^[97]. These results were confirmed in a similarly designed German RCT that included 131 patients. Survival in the statin group was 20.9 mo, 95%CI: 15.5-26.3 compared to 12.0 mo, 95%CI: 10.3-13.7, $P = 0.003$ in the control group^[98]. Similar data was reported from observational studies in Taiwan and United States. The Taiwanese authors observed 20200 patients who received palliative treatment for HCC with median follow-up of 1.66 years. Statin treatment in this group was associated with lower HCC-related deaths in all stages of HCC. The risk of HCC-related death was reduced in 50% during 18 mo' follow-up in patients with stage II and III^[99]. The American authors observed 1036 with early HCC (stage I or II) undergoing standard treatment for HCC. Patients who used statin lived significantly longer (23.9 vs 18.9 years, $P = 0.047$). However, after adjustment for confounders and immortal time bias, statin use did not confer lower risk of death (HR = 0.98, 95%CI: 0.80-1.20)^[100]. The reviewed studies suggest that the addition of statin to the treatment of patients with advanced HCC could extend survival by 5-9 mo. Surprisingly, the results of two RCTs are more favorable than the results of the SHARP study, where sorafenib treatment extended the survival of patients with non-

Table 1 Risk of overall and individual cancer with statin or metformin use in hepatitis B virus patients^[96]

All group (n = 71824)	No. of patients	Nonuser (n = 53037) Adjusted HR (95%CI)	Only-metformin (n = 4774) Adjusted HR (95%CI)	Only-statin (n = 8861) Adjusted HR (95%CI)	M + S (n = 5152) Adjusted HR (95%CI)
Total cancer	5434	1	1.03 (0.94-1.14)	0.60 (0.55-0.66) ^d	0.46 (0.40-0.52) ^d
Liver cancer	1735	1	1.25 (1.06-1.47) ^b	0.34 (0.27-0.42) ^d	0.35 (0.27-0.45) ^d
Nonliver cancer	3699	1	0.94 (0.83-1.06)	0.72 (0.65-0.80) ^d	0.50 (0.44-0.58) ^d
Lung cancer	439	1	0.91 (0.66-1.26)	0.51 (0.37-0.70) ^d	0.49 (0.34-0.71) ^d
Stomach cancer	144	1	0.77 (0.42-1.42)	0.59 (0.35-1.00) ^a	0.31 (0.14-0.69) ^b
Colorectal cancer	572	1	1.14 (0.85-1.53)	0.84 (0.65-1.09)	0.51 (0.35-0.75) ^d
Esophagus cancer	93	1	1.19 (0.61-2.31)	0.38 (0.17-0.86) ^a	0.30 (0.11-0.87) ^a
Pancreatic cancer	127	1	1.33 (0.74-2.41)	0.73 (0.40-1.31)	0.70 (0.34-1.43)
Prostate cancer	225	1	0.94 (0.59-1.50)	0.77 (0.51-1.15)	0.63 (0.37-1.05)
Breast cancer	288	1	0.80 (0.47-1.32)	0.91 (0.63-1.33)	0.56 (0.33-0.95) ^a
Cervical cancer	105	1	0.70 (0.31-1.58)	0.67 (0.35-1.25)	0.28 (0.10-0.79) ^a
Other cancers	1706	1	0.91 (0.76-1.09)	0.51 (0.42-0.64) ^d	0.75 (0.65-0.88) ^d

^aP < 0.05, ^bP < 0.01, ^dP < 0.001 *vs* control. Adjusted HR is adjusted for baseline propensity score. HBV: Hepatitis B virus; HR: Hazard ratio; M: Metformin; S: Statin.

resectable HCC by only 2.8 mo^[101].

LIMITATIONS OF STATIN USE IN PATIENTS WITH LIVER DISEASE

The conclusions that can be drawn from this review are limited by the mostly observational nature of the reviewed studies. However, the risk of bias seems to be relatively low because the control groups come from the same population as treated patients^[102]. Moreover, the evidence from RCTs is accumulating as well. There is not enough data to conclude if the various benefits of statins are related to the class effect, or if they are limited to particular a molecule (fluvastatin in chronic hepatitis B, simvastatin in portal hypertension, atorvastatin or fluvastatin in HCC risk reduction and pravastatin in palliative treatment of HCC).

A second concern about statins in liver disease is the potential hepatotoxicity. There are two possible reactions to the the statin treatment. The most common is an asymptomatic, dose-dependent increase of plasma transaminase activity. This is present in as much as 2.7% of all high dose statin users, and is also dependent on the particular statin molecule. For example, rosuvastatin has the least chance of causing the elevation of liver enzymes^[103]. The second possibility is a drug induced liver injury (DILI) that is a result of idiosyncratic reaction, dose independent, and has potentially serious consequences. The diagnostic criteria for DILI increased of ALT \geq 5-times above the upper limit of norm, or the increase of ALP \geq 2-times above the upper limit of norm. According to data from the Swedish Adverse Drug Reactions Advisory Committee, only 73 cases of DILI, two deaths and one liver transplant occurred over 23 years (1988-2010) of statin use in Sweden. That represents about 1.2 cases of DILI per 100000 users. The lowest rate of DILI was

reported for pravastatin and highest for fluvastatin^[104]. This rate is at the lower end of the range reported for general DILI incidence (from 1:10 000 to 1:100 000)^[105]. Despite this information, almost 50% of academic physicians hesitate to prescribe statin if ALT is greater than 1.5 times the upper limit of norm^[106].

Finally, the most common complication of statin treatment that leads to the statin treatment being stopped is drug-induced myopathy. This condition is associated with single nucleotide polymorphism in SLCO1B1^[48]. The risk of statin-induced myopathy can be lowered by the administration of coenzyme Q10 alone, or in combination with selenium^[107].

CONCLUSION

This review summarized the potential uses of statins in patients with various liver disease states. In patients with chronic hepatitis C the addition of statin improves SVR rates of PEG IFN treatment and slows down fibrogenesis,. While it is not clear if statins influence HCV RNA levels, the main benefit is in patients with advanced fibrosis or cirrhosis. Statins have the potential to decrease the rate of fibrosis possibly through the prevention of hepatic sinusoidal microthrombosis. Statins decrease HVP in patients with portal hypertension, and improve the survival of patients after variceal bleeding. Lower rates of infections were observed in patients with cirrhosis who received statin treatment. Statins decrease the risk of HCC in patients with advanced liver disease in general but particularly in patients with chronic hepatitis B and C. The addition of statin could prolong the survival of patients with advanced HCC. Most of the presented information comes from observational studies, randomized controlled trials are warranted to confirm these effects and allow the routine clinical use of statins in new indications.

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Hepatitis C virus - associated B cell non-Hodgkin's lymphoma

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Abstract

The hepatitis C virus (HCV) infected patients are prone to develop bone marrow or various tissue infiltrates with monoclonal B cells, monoclonal B lymphocytosis

or different types of B cell non-Hodgkin's lymphoma (BCNHL), of which the most common are splenic marginal zone BCNHL, diffuse large BCNHL and follicular lymphoma. The association between chronic HCV infection and non Hodgkin's lymphoma has been observed especially in areas with high prevalence of this viral infection. Outside the limitations of some studies that have been conducted, there are also geographic, environmental, and genetic factors that contribute to the epidemiological differences. Various microenvironmental signals, such as cytokines, viral antigenic external stimulation of lymphocyte receptors by HCV antigens, and intercellular interactions contribute to B cell proliferation. HCV lymphotropism and chronic antigenic stimulation are involved in B-lymphocyte expansion, as mixed cryoglobulinemia or monoclonal gammopathy of undetermined significance, which can progress to BCNHL. HCV replication in B lymphocytes has oncogenic effect mediated by intracellular HCV proteins. It is also involved in an important induction of reactive oxygen species that can lead to permanent B lymphocyte damage, as DNA mutations, after binding to surface B-cell receptors. Post-transplant lymphoproliferative disorder could appear and it has a multiclonal potentiality that may develop into different types of lymphomas. The hematopoietic stem cell transplant made for lymphoma in HCV-infected patients can increase the risk of earlier progression to liver fibrosis and cirrhosis. HCV infected patients with indolent BCNHL who receive antiviral therapy can be potentially cured. Viral clearance was related to lymphoma response, fact that highlights the probable involvement of HCV in lymphomagenesis. Direct acting antiviral drugs could be a solution for the patients who did not tolerate or respond to interferon, as they seem to be safe and highly effective. The use of chemotherapy in combination with rituximab for the treatment of BCNHL in patients infected with HCV can produce liver dysfunction. The addition of immunotherapy with rituximab can increase the viral replication, and severe complications can occur especially in patients co-infected with hepatitis B virus or immune immunodeficiency virus, in those with hepatocarcinoma,

cirrhosis, or liver cytolysis. But the final result of standard immunochemotherapy applied to diffuse large BCNHL patients with HCV infection is not notably worse than in those without this viral infection. The treatment of patients chronically infected with HCV and having BCNHL is complex and requires a multidisciplinary approach and the risk / benefit ratio of rituximab treatment must be evaluated especially in those with liver cytolysis.

Key words: Chemotherapy; Cryoglobulinemia; Direct acting antiviral drugs; Hepatitis C virus; Hepatocytolysis; Interferon; Liver transplantation; Liver dysfunction; Non-Hodgkin's lymphoma; Rituximab

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Core tip: There are epidemiological observations on the association between hepatitis C virus (HCV) infection and non-Hodgkin's lymphoma. Various microenvironmental signals, such as cytokines, viral antigenic external stimulation of lymphocyte receptors by HCV antigens, and intercellular interactions contribute to B cell proliferation. HCV lymphotropism and chronic antigenic stimulation are involved in B-lymphocyte expansion, as mixed cryoglobulinemia or monoclonal gammopathy of undetermined significance, which can progress to B cell non-Hodgkin's lymphoma (BCNHL). HCV infected patients with indolent BCNHL who receive antiviral therapy can be potentially cured. Viral clearance was related to lymphoma response, fact that highlights the probable involvement of HCV in lymphomagenesis.

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INTRODUCTION

The association between chronic hepatitis C virus (HCV) infection and some B cell non-Hodgkin's lymphomas (BCNHL) has been discussed for a long time^[1,2]. A higher incidence of these lymphomas has been found especially in countries where HCV prevalence is high (about 10%, according to a recent systematic review)^[3]. Unfortunately, this connection is not well understood. But, as it happens in many situations as the scientific research progresses and offers useful information, a possible pathway explanation has been recently published: a mutated stereotypic IGHV4-59/IGHJ5-encoded B-cell receptors subset was found to be expressed in some BCNHL associated with HCV infection. These mutated receptors are high affinity monoreactive rheumatoid factors and emphasize the auto-antigen role of IgG in BCNHL pathway^[4]. Given this news I decided to draw out a review on HCV-related

BCNHL. For this purpose I have studied the articles published in PubMed since January 2013 until today.

The temporal relationship between HCV infection and non-Hodgkin's lymphomas was analyzed in a large study conducted in Taiwan, after excluding patients infected with HCV who had cancers and infections with hepatitis B virus (HBV) or human immunodeficiency virus (HIV) at baseline. The follow-up of Asian patients infected with HCV established that their risk of developing a lymphoid neoplasm (and especially non-Hodgkin's lymphomas) was 2 times higher than that of a group of HCV-uninfected patients^[5].

EPIDEMIOLOGICAL DATA AND RISK FACTORS

The hepatic and extrahepatic manifestations of HCV are extremely varied as geographical distribution, fact which can be explained by a possible involvement of other environmental and/or genetic cofactors^[6].

The epidemiological studies made in the last 20 years found an association between HCV infection and BCNHL^[7]. Thus, Chronic Hepatitis Cohort Study followed a large group of patients with chronic HCV infection (12126 subjects) during 5 years and found the following values: the incidence of non-Hodgkin's lymphoma was significantly higher [standardized rate ratios was 1.6 (1.2-2.1)], and age-adjusted mortality was also significantly higher than the general population^[8].

Indolent non-Hodgkin's lymphomas were more frequently associated with HCV infection^[9]. Indeed, HCV infection was more frequently found in patients with marginal zone lymphomas, and especially in those with splenic type, compared to the control population, an argument for a possible viral involvement in lymphoma genesis^[10]. A large meta-analysis which included 2440 patients with small lymphocytic lymphoma and chronic lymphocytic leukemia confirmed the association of these hemopathies with HCV infection^[11]. But in a cohort of 524 Bulgarian patients with non-Hodgkin's lymphoma, only 1.84% were HCV positive^[9]. Patients with dual viral infection - HCV and HIV - are also more likely to develop marginal zone/lymphoplasmacytic BCNHL, compared to HIV only-infected patients^[12].

The association between HCV infection and indolent BCNHL has been known for a long time, but this virus infection can also be associated with diffuse large BCNHL, especially in some geographical regions^[13], so it is considered that marginal zone lymphomas and diffuse large BCNHL are the histological type commonly associated with this viral infection^[14]. Indeed, the most frequent type of BCNHL found in 89 HCV infected patients was that with large cells (62%) in a study realized at MD Anderson Cancer Center during 7 years. Their liver disease was mostly mild (only 18% of patients had a Metavir stage ≥ 3), the most frequent genotype was 1 (62%), and viremia was detected in 90% of patients^[2]. HCV

infection was more frequently found in patients with splenic diffuse large BCNHL and splenic marginal zone BCNHL compared to patients with all types of lymphoma in Italy, while the prevalence of this virus was higher only in those with diffuse large BCNHL compared to the patients with all types of lymphoma, in Japan. Forty-four percent of patients with diffuse large BCNHL and 10% of those with splenic marginal zone BCNHL were HCV positive in a study conducted in Taiwan^[15]. In ANRS HC-13 Lympho-C study which included 116 HCV infected patients with BCNHL, the most frequent histological types were marginal zone lymphoma and diffuse large BCNHL (both present in 39% of patients)^[16].

Beside splenic marginal zone BCNHL, diffuse large BCNHL and follicular lymphoma, HCV chronically infected patients can develop a disseminated type of marginal zone lymphoma with different characters from splenic marginal zone lymphoma or a monoclonal B lymphocytosis and bone marrow or various tissue infiltrate with monoclonal B cells, without histology of lymphoma^[17]. A higher risk for B-cell activating autoimmune conditions was found to be associated with all 3 subtypes of marginal zone BCNHL (nodal, extranodal and splenic), but HCV infection was a risk factor only for the extranodal subtype, vs the witnesses^[18].

A large meta-analysis established that the meta-lworker occupation, the presence of hematologic neoplasias in the family history, and the patient-declared peptic ulcers are risk factors for the extranodal subtype of marginal zone BCNHL. On the contrary, a reduced risk for this subtype of lymphoma was present in teachers and those who drank any kind of alcohol^[18].

The diffuse large BCNHL was associated with HCV infection, but also with B-cell activating autoimmune disorders, the presence of non-Hodgkin lymphoma in the family history, a body mass index at young adult age, any atopic disturbance, higher socioeconomic status, and higher sun exposure in free time, according to another large meta-analysis^[19].

The patients with non-Hodgkin's lymphoma have an 1.5 times higher risk for a second primary malignancy occurrence, most commonly for leukemia and myeloma. Liver cirrhosis and HCV infection were significant predictors for the appearance of such a new malignancy in a retrospective study made in Taiwan^[20].

PATOPHYSIOLOGY NOTIONS

Some cases of non-Hodgkin's lymphoma might be due to HCV infection, particularly in areas with high prevalence of this infection^[1,2,21]. In the early-stage of diffuse large BCNHL HCV seroprevalence was high in a study conducted in Taiwan, fact which advocates for the involvement of HCV in lymphoma pathway^[15].

A question remains unanswered: why didn't some studies find any association between HCV infection

and lymphoma. It is considered that the small number of patients, the short follow-up period analyzed and database limitations have influenced the results of these studies^[21]. In addition, epidemiological studies used mainly anti-HCV antibody test, which has lower sensitivity compared to HCV-RNA detection, the most widely used test in order to detect the association between HCV and non-Hodgkin's lymphoma^[7].

Among lymphoproliferative diseases, BCNHL appears to be mostly associated with HCV infection^[21]. It is known that HCV has both hepato- and lymphotropism and is involved in a polyoligoclonal B-lymphocyte expansion, followed sometimes by the occurrence of mixed cryoglobulinemia (an immune-mediated disorder)^[6]. Both chronic inflammation and alterations in immune function, are involved in B cell lymphoproliferative disorders^[21], as mixed cryoglobulinemia or monoclonal gammopathy of undetermined significance^[22]. These may progress to BCNHL^[6,21] (Figure 1). Therefore, it can be considered that the evolution of the malignant clone is the result of both HCV lymphotropism and chronic antigenic stimulation^[23]. The presence of cirrhosis in HCV infected patients is a supplementary risk factor for monoclonal gammopathy of undetermined significance or BCNHL occurrence, in agreement with a multivariate logistic regression analysis^[22].

Regarding the histological subtypes of BCNHL, the rheumatoid factor was more frequently noticed in patients with marginal zone lymphoma, than in those with diffuse large B-cell (68% compared to 35%). Mixed cryoglobulinemia was also significantly more often found in the first mentioned subtype (74% vs 44%)^[16]. There are arguments in favor and against the association of chronic HCV infection and Waldenström macroglobulinemia. The HCV potential to promote lymphoproliferation is supported by some authors, as in the case of an HCV-infected patient with cryoglobulinemia and clinical manifestations of hyperviscosity, where bone marrow biopsy established the diagnosis^[24]. In HCV-infected patients with cryoglobulinemia only memory B cells, not naïve cells, were found to be significantly activated compared to healthy subjects. Markers of these cell activation in patients with these associated pathology were CD71, CD86, and HLA-DR, and in those with advanced hepatic disease - CD86^[25].

The association of BCNHL with HCV infection is not completely defined although epidemiological studies argued for its support^[14].

The mechanism of lymphomagenesis should be considered. It is known that infectious, environmental, and genetic factors are involved in this multifactorial process^[21]. As mentioned above, some laboratory and clinicoepidemiological studies have suggested the oncogenic potential of HCV^[6]. It is believed that lymphomagenesis depends on chronic rather than cleared HCV infection^[7].

Usually, the malignant cells coexist with the

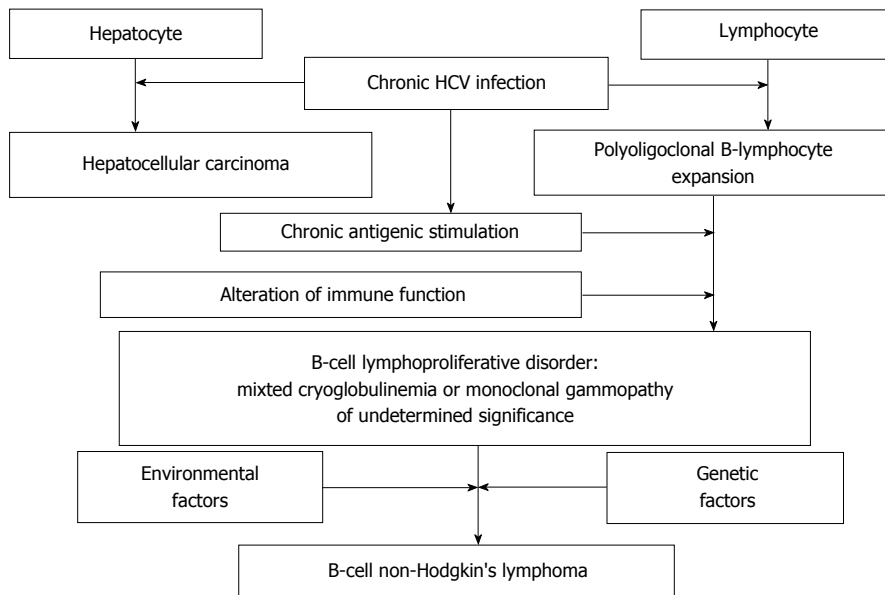


Figure 1 Main mechanisms of lymphomagenesis. HCV: Hepatitis C virus.

microenvironmental factors. At least at the beginning, lymphoma development depends on various micro-environmental signals, such as: cytokines, viral antigens, and intercellular interactions^[14]. Continuous viral antigenic external stimulation of lymphocyte receptors by HCV seems to be of major importance for B cell proliferation^[14,26]. The regression of BCNHL after HCV infection eradication with antiviral treatment is another argument for the virus involvement in BCNHL pathway^[26].

It is known that HCV-associated lymphomas use a restricted immunoglobulin variable region gene repertoire, so that the lymphoma B-cell receptors expressed as soluble immunoglobulin Gs and membrane IgMs do not bind to the HCV antigens. It follows that the majority of lymphomas do not occur from B cells that are involved in viral clearance^[27].

Another theory states that HCV replication in B lymphocytes has oncogenic effect mediated by intracellular HCV proteins^[26].

Another mechanism would be the one in which the HCV is involved in an important induction of a reactive oxygen species^[28] and can lead to permanent B lymphocyte damage, as DNA mutations of tumor suppressor gene^[26] TP53 and proto-oncogenes CTNNB1 and BCL6^[29] and/or lower antigen response thresholds, after binding to surface B-cell receptors^[21]. Still, a study made on 6 HCV-infected patients did not find any suspected mutation, so the authors concluded that HCV does not generally induce mutations in the genes involved in oncogenesis, as CTNNB1, TP53, and BCL6 in B lymphocytes^[29]. Moreover, it was found that cytotoxic T-lymphocyte antigen 4 (CTLA-4) + 49 A/G polymorphism is associated with a higher risk of BCNHL occurrence. HCV infection was more frequently present in subjects carriers of the mutant genotype + 49 A/G and - 318 C/T SNPs was more often found in

patients with BCNHL and was a risk factor for BCNHL occurrence^[30].

Interleukin 28B gene polymorphisms also seems to be involved in lymphomagenesis. Thus, the IL-28B C/C genotype is distinguished biologically by a higher frequency of restriction of B cell response and its presence is correlated with a higher probability of cryoglobulinemic nephropathy and B cell malignant proliferations^[31].

A deregulation of NF- κ B, NOTCH, and BCR signaling pathway can be found to arise in the pathogenesis of splenic marginal zone lymphoma. But there is evidence that NOTCH pathway lesions are significantly more frequently found in HCV-infected patients with diffuse large BCNHL as compared to patients with the same type of lymphoma but uninfected with HCV. In addition, those who had a NOTCH pathway mutation had a significantly shorter 5-year overall survival vs the patients without lesions in NOTCH pathway (27% compared to 62%)^[32]. Indeed, both canonical and alternative NF- κ B signalling pathways were activated and miR-26b expression was down-regulated in a transgenic mice model that express the full-length HCV genome specifically in B cells and which developed an HCV-associated BCNHL^[33].

An area of particular interest on lymphomagenesis in HCV-infected patients is the study of microRNA levels in lymphoma tissues. Thus, an increased expression level of miR-30b in 14 biopsies from HCV- and HBV-infected patients with indolent BCNHL was observed. An association between miR-29a, miR-29b, and miR-223, and the presence of HCV infection was found in patients with nodal marginal zone lymphoma^[34]. Regarding the HCV-infected patients with diffuse large BCNHL, a set consisting of 52 miRNAs could be a signature for them. It should also be noted that miR-138-5p which had a decreased

expression, and miR-511-5p, miR-147a, and miR-147b which had an increased expression serve as a negative prognostic factor in HCV-infected patients with diffuse large BCNHL^[35]. Further research is needed in order to establish the role of microRNA over- or underexpression in BCNHL pathogenesis and their usefulness in a possible new classification of lymphomas.

The t(14;18) translocation has been found in B lymphocyte proliferation and it is considered to be associated with MALT lymphomas occurrence in HCV-infected patients^[36]. A more frequent telomeric 1p36.3 deletion and an increased expression of Ki-67 were found in BCNHL patients with HCV infection vs those without this infection. This is an argument for a possible virus involvement in cancers occurrence at the 1p36.3 locus^[37].

Oncogenic potential of HCV can lead to a concomitant induction of hepatocellular carcinoma and a BCNHL, as in a case of synchronous neoplasia found during the process of liver transplantation surgery^[38].

INFLUENCE OF LIVER TRANSPLANTATION

Post-transplant lymphoproliferative disorder could appear and it has a multiclonal potentiality that may develop into different types of lymphomas^[39].

In a large study which included 10010 Swedish patients with a solid organ transplant, 135 patients developed a lymphoma. The incidence rate of lymphomas was 159/100000 person-years. Forty-eight percent of them were negative for Epstein-Barr virus (EBV) infection and associated with HCV infection. HCV infection was found to be an independent negative prognostic factor for survival in these patients^[40].

In such a patient who underwent liver transplantation for hepatitis C liver cirrhosis, developed a relapse of his hepatitis C 2 mo after the graft. An EBV-negative polymorphic B-cell and an EBV-negative monomorphic T-cell ALK-positive post-transplant lymphoproliferation occurred after another 32 mo. They were treated with R-CHOP regimen followed by complete remission. However, the lymphoproliferation recurred and the patient died half a year after the first post-transplant lymphoproliferative diagnosis. Histopathological examination of the liver and other organs discovered a third type of lymphoproliferation: an EBV-negative monomorphic T-cell ALK-negative lymphoma^[39]. Immunosuppression, but also relapsed HCV infection are involved in the pathogenesis of these post liver transplant lymphoproliferations. A primary hepatic diffuse large BCNHL was also histologically diagnosed in an HCV-infected patient who had undergone a partial hepatectomy. After he was treated by systemic chemotherapy, the patient developed a new liver tumor, which was also operated by partial hepatectomy and was a hepatocellular carcinoma^[41].

The hematopoietic stem cell transplant made for lymphoma in HCV-infected patients can increase the risk of earlier progression to liver fibrosis and cirrhosis. At this point, it is unknown whether this risk is or is not dependent of prior treatment of lymphoma^[42].

CLINICAL AND LABORATORY FINDINGS

The clinical manifestations of diffuse large BCNHL are not the same in HCV-positive or negative patients^[13]. Indeed, it seems that liver involvement is greater and the number of affected nodal regions is higher in patients with diffuse large BCNHL with HCV/HBV infection as in those without such viral infections, according to the results of a study which included 224 diffuse large BCNHL patients but of which only 9.3% were HCV/HBV-positive. Despite these differences, identically treated patients had similar responses and evolution in the study made by Rubio *et al*^[43].

Sometimes, HCV-infected patients can develop BCNHL with various locations. In such a patient who had liver cirrhosis with double etiology (alcoholic, too) a primary follicular lymphoma appeared in the spleen was found when he underwent a splenectomy performed in order to reduce symptomatic pancytopenia^[44]. Another rare location is on the skin. A localized lipomatrophy was reported to be a clinical manifestation of a marginal zone BCNHL^[45]. A primary large BCNHL was also found in the body cavity of an HCV-infected patient^[46] and a marginal zone lymphoma located on the right lacrimal gland associated to an HCV-infection was also reported^[47].

As lymphoma patients are immunosuppressed, RNA detection techniques of HCV infection are more frequently requested compared to the other patients^[42], in order to detect early this possible infection and to avoid a possible viral flare induced by chemotherapy.

Based on a multiparametric analysis, four serum parameters were identified in order to constitute a signature able to differentiate the HCV-infected patients with or without overt BCNHL, with a sensitivity of 100% and a specificity of 90%: sCD27, C4 levels, sIL-2Rα, and gammaglobulins^[48].

Mixed cryoglobulinemia (MC) was found in about a quarter of a large cohort of patients with chronic HCV infection and three quarters of those with MC had also cryoglobulinemic syndrome. These patients presented BCNHL significantly more often than those without MC (15% as against 7.1%). If cryoglobulinemic syndrome had no impact on the overall survival, it would modify the natural history of these patients, as shown in a 15-years prospective cohort study^[49].

The patients with BCNHL would have a shorter overall survival if they were HCV-infected and had 1p36.3 deletion found by FISH, vs those without HCV infection (37). Three risk factors which correlated with a bad prognosis in HCV-infected patients with diffuse large BCNHL were found in a multivariate analysis: an

Table 1 Main therapeutical findings

Study findings	Ref.
Antiviral treatment should be indicated in order to prevent lymphoma occurrence	[2]
Low-grade malignant lymphomas can respond to antiviral therapy	[26,51]
Non-Hodgkin's lymphoma with high grade of malignancy need immuno-chemotherapy associated treatment	[7,15]
Antiviral treatment contributed to an improved outcome of HCV-infected patients with non-Hodgkin's lymphoma	[16]
Antiviral treatment could be an alternative to chemo-immunotherapy in some cases	[23]
Splenic marginal zone lymphoma is most frequently associated with HCV infection and can evolve favorably after HCV eradication	[53]
HCV-infected patients with indolent BCNHL who receive antiviral therapy can be potentially cured	[13]
Forty-four of HCV-infected patients with indolent BCNHL obtained a complete remission and 33% a partial response of lymphoma after antiviral therapy used as first-line treatment	[55]
Viral clearance was related to lymphoma response	[55]
The clinical response of lymphoma is dependent on HCV-RNA eradication	[14]
The combined treatment with peginterferon and ribavirin proved to be useful for the treatment of BCNHL	[56]
Repeated plasmapheresis are needed, if hyperviscosity is present, followed by antiviral \pm cytostatic therapy	[24]
The administration of direct antiviral agents is useful in onset of therapy of patients with marginal zone BCNHL who have no severe complications, and early in those with diffuse large BCNHL in order to prevent the potential liver damage induced by the use of immunochemotherapy and avoid BCNHL relapse	[56]
A chronic HCV-infected patient with splenic marginal zone lymphoma obtained rapid viral clearance and his lymphoma was cured with an interferon-free regimen based on NS3-NS4A inhibitor	[57]
A HCV-infected female patient with chronic lymphocytic leukaemia received telaprevir-based triple therapy followed by successful result, without chronic lymphocytic leukaemia progression	[58]
HCV-infected diffuse large BCNHL patients had a higher liver toxicity induced by immunochemotherapy and a higher delay of their chemotherapy application	[59]
Severe liver toxicity (grade 3-4) was significantly more frequently found in diffuse large BCNHL patients infected with HCV treated also by immunochemotherapy compared with those treated only by chemotherapy	[60]
The liver toxicity of grade 3-4 was significantly more frequently found in HCV-infected patients with diffuse large BCNHL treated with chemo-immunotherapy and the progression-free survival and overall survival were significantly shorter in comparison with those who received only chemotherapy	[61]
Fourteen percent of HCV-infected patients with diffuse large BCNHL who received an anthracycline-based chemotherapy (with rituximab in 255 of them) developed severe liver toxicity	[50]
A patient with diffuse large BCNHL and HCV infection developed a cholestatic hepatitis C after chemoimmunotherapy	[62]
The addition of immunochemotherapy with rituximab can increase the viral replication	[13]
The final result of standard immunochemotherapy applied to diffuse large BCNHL patients with HCV infection is not less good compared to those without this infection	[13]
A solution to avoid a severe liver toxicity in patients with compensated HCV induced liver cirrhosis and indolent BCNHL is the combination of bendamustine with rituximab	[63]

HCV: Hepatitis C virus; BCNHL: B cell non-Hodgkin's lymphoma.

ECOG performance status ≥ 2 , serum level of albumin < 3.5 g/dL, and HCV-RNA viremia > 1000 KIU/mL. A score which includes these three factors could be used to discriminate the patients with different overall and progression-free survival, independent of their treatment (with or without rituximab)^[50].

THERAPEUTIC PARTICULARITIES

Classical antiviral therapy

The treatment of both HCV infection and lymphoma is a challenge for physicians. The main findings in this field are presented in Table 1. It is a pity that not all cancer patients can benefit from antiviral therapy^[42]. In addition, we do not know which is the best course of treatment in BCNHL patients chronically infected with HCV, currently^[51]. Although 53 patients of the study realized at MD Anderson Cancer Center were detected with HCV infection before the diagnosis of non-Hodgkin's lymphoma (which was made later), almost half of them were not treated with antiviral medication, especially as they had mild liver disease at diagnosis^[2]. As HCV infection can be involved in cancer occurrence, including hepatocellular carcinoma and

non-Hodgkin's lymphoma^[42], early antiviral treatment should also be indicated for a possible prevention of lymphoma occurrence^[2].

Low-grade malignant lymphomas can respond to antiviral therapy^[26,51] if the disease is limited and do not require immediate cytoreductive drugs^[52]. Those with high grade of malignancy need also immuno-chemotherapy associated treatment^[7,51], despite the probable liver toxicity^[7].

It was shown that antiviral treatment contributed to an improved outcome of HCV-infected patients with non-Hodgkin's lymphoma^[16] and, in some cases, could also be an alternative to chemo-immunotherapy^[23]. Of the three types of marginal zone lymphoma (MALT, nodal and splenic), the last is most frequently associated with HCV infection and can evolve favorably after HCV eradication^[53]. A rare association between BCNHL and mixed cryoglobulinemic endocapillary proliferative glomerulonephritis found sometimes in HCV-infected patients can have a favourable evolution under interferon: such a patient had a reduction of its clinical symptoms, proteinuria disappeared and HCV viremia decreased after one year of treatment^[54].

HCV-infected patients with indolent BCNHL who receive antiviral therapy can be potentially cured^[13]. Forty-four of HCV-infected patients with indolent BCNHL showed complete remission and 33% a partial response of lymphoma after antiviral therapy used as first-line treatment, in a large multicenter study^[55]. As it is known that sustained virological responses to HCV antiviral treatment in cancer patients may be poorer as in those without cancer^[42], it is very important to note that viral clearance was related to lymphoma response in this multicenter study^[55]. One can speculate that the persistence of virus in malignant lymphocytes could constitute a reservoir that can contribute to hepatitis relapse. On the other hand, the clinical response of lymphoma is dependent on HCV-RNA eradication, fact that highlights the probable involvement of HCV in lymphomagenesis^[14].

The combined treatment with peginterferon and ribavirin proved to be useful for the treatment of BCNHL^[56]. The fact that antiviral treatment may be followed by complete remission of lymphoma is an argument for a possible involvement of chronic antigenic stimulation and HCV in BCNHL pathway^[51].

If hyperviscosity is present, as in HCV-infected patients with IgM or IgG gammopathy, or Waldenström's macroglobulinemia, repeated plasmapheresis are needed in order to fight against this syndrome, followed by antiviral ± cytostatic therapy^[24].

Direct acting antiviral therapy

Direct acting antiviral drugs could be a solution for the patients who did not tolerate or respond to interferon, as they are safe and highly effective, according to recent findings^[51,57]. Indeed, five cases of BCNHL patients infected with HCV obtained sustained virological response after direct anti-viral agents therapy, given alone, together with rituximab or followed by chemotherapy. Four of them achieved complete remission of BCNHL 6 mo after the treatment ended. These results suggest the administration of direct antiviral agents in onset of therapy of patients with marginal zone BCNHL who have no severe complications, and early in those with diffuse large BCNHL in order to prevent the potential liver damage induced by the use of chemotherapy in combination with rituximab and avoid BCNHL relapse^[56]. A chronic HCV-infected patient with splenic marginal zone lymphoma obtained rapid viral clearance and his lymphoma was cured with an interferon-free regimen based on NS3-NS4A inhibitor, which consisted in a 16 wk administration of sofosbuvir, faldaprevir, and ribavirin. Such therapeutic results, achieved even with interferon free regimens highlight the pathogenetic role of the virus in the development of lymphoma and also suggest that the effectiveness of interferon therapy of lymphoma is especially due to its antiviral and less antiproliferative effect^[57].

The case of a patient who obtained a haematological

response after peginterferon plus ribavirin was also published. Still, a virological relapse was noted at week 24, for which she received telaprevir-based triple therapy, followed by successful result without chronic lymphocytic leukaemia progression^[58].

We hope that the era of interferon-free regimen will also bring clarifications on the importance of the lymphoid reservoir in HCV removal^[23].

Rituximab-based chemotherapy

There is a debate on the safety of rituximab-based chemotherapy used for the treatment of diffuse large BCNHL. Twenty-nine HCV-infected patients with this type of lymphoma were compared with 139 patients without HCV infection but with the same type of lymphoma. HCV-infected patients had a higher liver toxicity induced by immunochemotherapy (manifested in particular by an increase of AST and total bilirubin) and a higher delay of their chemotherapy application, without affecting survival, during a median follow-up of 3 years^[59]. In another study, 200 diffuse large BCNHL patients infected with HCV were treated with chemotherapy combined with rituximab, vs 80 patients with the same two diseases who received only chemotherapy. There were no significant differences on median progression-free survival or median overall survival, but severe liver toxicity (grade 3-4) was significantly more frequently found in those treated also by immunochemotherapy compared with those treated only by chemotherapy (26.5% vs 13.75%). A quarter of patients who received rituximab could not complete the therapy due to liver toxicity or their progressive disease. A risk factor predictive for severe liver toxicity was the presence of liver dysfunction before the treatment^[60]. The results were worse in another study, which included 137 HCV-infected patients with diffuse large BCNHL treated with CHOP ± rituximab regimen. The liver toxicity of grade 3-4 was significantly more frequently found in those treated with chemo-immunotherapy (28% vs 18%), while the progression-free survival and overall survival were significantly shorter in this group of patients in comparison with those who received only chemotherapy^[61]. In a larger study, made on 535 HCV-infected patients with diffuse large BCNHL who received an anthracycline-based chemotherapy (with rituximab in 255 of them), 14% of patients developed severe liver toxicity, but, in this study, rituximab did not contribute to an increased severe liver toxicity. Overall survival and progression-free survival at 3 years were 71% and, respectively, 55%^[50].

Therefore, the use of chemotherapy in combination with rituximab (an anti-CD20 monoclonal antibody) for the treatment of BCNHL in patients infected with HCV can produce liver dysfunction (as adverse effect), like the chemotherapy applied for Hodgkin's lymphoma cure. A rare case of cholestatic hepatitis C was also published; it occurred in a patient with diffuse large BCNHL and

HCV infection^[62]. The addition of immunotherapy with rituximab can increase the viral replication, but severe complications can occur especially in patients co-infected with HBV or immune immunodeficiency virus, in those with hepatocarcinoma, cirrhosis, or liver cytolysis (an increase of transaminases of grade > 2). There is not necessarily a direct association between the level of HCV viremia and the liver lesions^[13]. The final result of standard immunochemotherapy applied to diffuse large BCNHL patients with HCV infection is not less good compared to those without this viral infection^[13]. A solution to avoid severe liver toxicity in patients with compensated HCV induced liver cirrhosis and indolent BCNHL is the combination of bendamustine with rituximab^[63].

CONCLUSION

There are strong arguments on the association between chronic HCV infection and BCNHL.

HCV lymphotropism and chronic antigenic stimulation are involved in B-lymphocyte expansion, as mixed cryoglobulinemia or monoclonal gammopathy of undetermined significance, which can progress to BCNHL^[6,21].

Classical or direct acting antiviral therapy can help cure HCV-infected patients with indolent BCNHL. This also highlights the probable involvement of HCV in lymphomagenesis^[14].

The use of chemotherapy in combination with rituximab for the treatment of BCNHL in HCV-infected patients can produce liver dysfunction.

The treatment of patients chronically infected with HCV and having BCNHL is complex and requires a multidisciplinary approach: a hematologist and a hepatologist should also be invited to participate. A careful monitoring of hepatic function is necessary^[13,51].

What is the best conduct in front of BCNHL patients (and especially of those with large cells) with liver cytolysis, what is the risk/benefit ratio of rituximab treatment, and what are the conditions in which we need to start or stop the immuno-chemotherapy are topics to which we expect future answers from the scientific research^[13].

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Basic Study

Serum metabolome profiles characterized by patients with hepatocellular carcinoma associated with hepatitis B and C

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Abstract

AIM: To clarify the characteristics of metabolite profiles in virus-related hepatocellular carcinoma (HCC) patients using serum metabolome analysis.

METHODS: The serum levels of low-molecular-weight metabolites in 68 patients with HCC were quantified using capillary electrophoresis chromatography and mass spectrometry. Thirty and 38 of the patients suffered from hepatitis B virus-related HCC (HCC-B) and hepatitis C virus-related HCC (HCC-C), respectively.

RESULTS: The main metabolites characteristic of HCC were those associated with glutathione metabolism, notably 13 γ -glutamyl peptides, which are by-products of glutathione induction. Two major profiles, *i.e.*, concentration patterns, of metabolites were identified in HCC patients, and these were classified into two groups: an HCC-B group and an HCC-C group including some of the HCC-B cases. The receiver operating characteristic curve for the multiple logistic regression

model discriminating HCC-B from HCC-C incorporating the concentrations of glutamic acid, methionine and γ -glutamyl-glycine-glycine showed a highly significant area under the curve value of 0.94 (95%CI: 0.89-1.0, $P < 0.0001$).

CONCLUSION: The serum levels of γ -glutamyl peptides, as well as their concentration patterns, contribute to the development of potential biomarkers for virus-related HCC. The difference in metabolite profiles between HCC-B and HCC-C may reflect the respective metabolic reactions that underlie the different pathogeneses of these two types of HCC.

Key words: Metabolome; Hepatocellular carcinoma; γ -glutamyl peptides; Glutathione; Oxidative stress

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Core tip: Serum metabolome analysis was applied to the patients of hepatocellular carcinoma (HCC) infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) to clarify the characteristics of their metabolite profiles. This study demonstrated that the serum concentrations of γ -glutamyl peptides and their concentration patterns contribute to the development of potential biomarkers for virus-related HCC. The difference in metabolite profiles between the HCC patients infected with HBV and HCV may reflect the respective metabolic reactions that underlie the different pathogeneses of these two types of HCC.

Saito T, Sugimoto M, Okumoto K, Haga H, Katsumi T, Mizuno K, Nishina T, Sato S, Igarashi K, Maki H, Tomita M, Ueno Y, Soga T. Serum metabolome profiles characterized by patients with hepatocellular carcinoma associated with hepatitis B and C. *World J Gastroenterol* 2016; 22(27): 6224-6234 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6224.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6224>

INTRODUCTION

Chronic hepatitis due to hepatitis C virus (HCV) or hepatitis B virus (HBV) causes progressive liver inflammation, which may eventually lead to liver cirrhosis and hepatocellular carcinoma (HCC)^[1,2]. The inflammatory process in hepatitis is closely associated with the oxidative stress induced by reactive oxygen species (ROS). Healthy individuals have their own protective mechanisms against oxidative stress, *i.e.*, the induction of anti-oxidative substrates, such as glutathione, thioredoxin, vitamin A and vitamin E, or enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, to remove ROS; however, these mechanisms are impaired in patients with

chronic hepatitis B or C, and thus, the long-term exposure to oxidative stress during viral infection leads to progressive liver disease accompanied by a risk of HCC^[3-9].

Metabolome analysis has been used to identify novel biomarkers for various liver diseases including HCC, based on the use of liquid chromatography and mass spectrometry (MS)^[10,11] or nuclear magnetic resonance and MS^[12-14]. In previous studies using capillary electrophoresis (CE) and MS^[15,16], we showed that ophthalmate (γ -glutamyl-2-aminobutyrylglycine) was applicable as a biomarker of reduced glutathione depletion in mice with acetaminophen-induced hepatotoxicity. Furthermore, we have demonstrated that the serum levels of γ -glutamyl dipeptides (γ -Glu-X) were increased in the majority of patients with nine types of liver disease^[17]. As γ -glutamyl dipeptides are synthesized *via* ligation of glutamine with various amino acids and amines by γ -glutamylcysteine synthetase, which is under feedback inhibition by glutathione, the level of γ -glutamyl dipeptides represents the degree of glutathione production. Therefore, γ -glutamyl dipeptides may represent key metabolites that reflect the extent of liver tissue injury due to oxidative stress.

In our previous study^[17], we also evaluated the potential use of γ -glutamyl dipeptides for diagnosis of HCC, and found that multiple logistic regression (MLR) models employing four γ -glutamyl dipeptides (γ -Glu-Ala, γ -Glu-Citrulline, γ -Glu-Thr, and γ -Glu-Phe) were able to distinguish HCC from chronic hepatitis and cirrhosis. Furthermore, anti-viral therapy for chronic hepatitis C using pegylated interferon plus ribavirin, which is known to reduce the risk of HCC development^[18-21], has been reported to improve the serum levels of γ -glutamyl dipeptides in treated patients^[22]. Thus, γ -glutamyl dipeptides have the potential to become biomarkers for HCC, and monitoring of their serum levels may be useful for the prediction of HCC occurrence.

Although chronic HBV or HCV infection may lead to HCC as an end-stage liver disease, the inflammatory process during chronic viral infection, which finally results in HCC, might differ between HBV and HCV infections. In fact, a genomic study of gene expression in liver tissues chronically infected with HBV and HCV demonstrated that the genes expressed predominantly were those related to inflammation and the anti-inflammatory response, respectively^[23]. It still remains unknown whether the serum metabolite profiles differ between patients with HBV-related HCC (HCC-B) and those with HCV-related HCC (HCC-C).

In the present study, we conducted a metabolome analysis of serum samples of HCC-B and HCC-C using CE-MS/MS. It was anticipated that this might lead to the development of a useful biomarker for virus-related HCC in clinical practice, and yield valuable information on differences in metabolite profiles between HCC-B

and HCC-C.

MATERIALS AND METHODS

Patients and sample collection

Sixty-eight patients who had been diagnosed for the first time as having HCC had chronic HBV or HCV infection, comprising 50 men and 18 women, with an age range of 41 to 87 years (68.6 ± 11.5 years, mean \pm SD). HCC patients without chronic viral infection such as those having alcoholic liver injury, autoimmune liver disease or metabolic liver disease were excluded. Each patient's serum was collected as early as possible following the date of HCC diagnosis; the mean date of serum collection was 33.8 d after the date of HCC diagnosis. Fifty-three (78%) of the serum samples were collected within 30 d after diagnosis. The samples were kept in clean tubes and stored at -80°C . The metabolome analysis was carried out at the Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan. This study was approved by the institutional ethics committee, and all patients gave their informed consent.

Serum metabolome analysis

Treatment of samples and quantification of metabolites: Following centrifugation for 10 min at $2000 \times g$, the serum samples were collected and stored at -80°C until measurement. Prior to metabolome analysis, the samples (40 μL) were added to 400 μL of methanol containing internal standards, together with methionine sulfone (Wako, Osaka, Japan), D-camphor-10-sulfonic acid (Wako), and 2-(n-morpholino)ethanesulfonic acid (Dojindo, Kumamoto, Japan), each at 20 $\mu\text{mol/L}$. Then 120 μL of Milli-Q water (Millipore, Billerica, MA) and 400 μL of chloroform were added, and the solution was centrifuged at $10000 \times g$ for 3 min at 4°C . The 300 μL upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter to remove large molecules, and the filtrate was dried by centrifugal concentration for 2 h at 40°C . Finally, it was dissolved in 20 μL Milli-Q water containing reference compounds (3-aminopyrrolidine and trimesate at 200 $\mu\text{mol/L}$ each). The γ -glutamyl peptides, glucosamine, and reduced and oxidized forms of glutathione were quantified using CE-MS/MS. The other metabolites were quantified using capillary electrophoresis-time-of-flight mass spectrometry, following the procedures previously described^[22] using different version of software; Agilent ChemStation software (B.04.03) and Agilent MassHunter (B.04.00 for anion and B02.01 for cation).

Profiling technique for serum metabolites using

CE-MS/MS: The metabolites were separated in a fused silica capillary (50 μm i.d. \times 100 cm) filled with 200 mmol/L ammonium acetate (pH 3.3) as

the run buffer^[15]. Prior to each run, the run buffer was injected for 8 min as preconditioning. A sample solution was injected at 50 mbar for 10 s (10 nL), and a voltage of 30 kV was applied. The capillary temperature and sample tray were set at 20°C and below 5°C , respectively. The mass spectrometer was set to run a multi-channel reaction, while monitoring in positive ion mode. The sheath liquid comprised 0.5 mmol/L ammonium acetate in methanol/water (50% v/v) delivered at 10 $\mu\text{L/min}$. The flow rate of heated dry nitrogen gas (heater temperature, 300°C) was maintained at 10 L/min. The capillary voltage was set at 4 kV. The pressure and flow rate of the nebulizer gas were 7 psi and 10 $\mu\text{L/min}$, respectively. Exact mass data were acquired at a rate of 1.5 spectra/s. Mass values for precursor and productions, fragmenter voltage, and collision energy were optimized for the individual metabolites. AS the CE-MS/MS instrument, we used a G1600AX Agilent CE capillary electrophoresis system (Agilent), an Agilent 1100 series binary HPLC pump, a G1948B ion source, a G1607A Agilent CE-ESI-MS sprayer kit, and an Agilent 6410 series Triple Quad. Data were acquired with Agilent ChemStation software (A.09.03) and Agilent MassHunter (Data Acquisition for Triple Quad B.04.01). Metabolite identification was conducted by matching the m/z values and corrected migration times^[24]. To quantify individual metabolites, commercially available standards were analyzed prior to sample analysis. The peak areas of all metabolites were divided by the peak area of the internal standard and compared with the standard compounds to calculate the concentrations. Raw data were processed using MasterHands^[25].

Statistical analysis

The Mann-Whitney *U*-test, Wilcoxon matched-pairs signed rank test and Fisher exact test were used to assess the statistical significance of differences at a significance level of $P < 0.05$. Heat map visualization was performed to indicate the similarities of metabolites and samples. Hierarchical clustering was conducted by Pearson correlation, and only metabolites showing $P < 0.05$ (Mann-Whitney *U*-test) between HCC-B and HCC-C were used. The colors on the heat map were determined by the Z-score of the metabolite concentration. Receiver operating characteristic (ROC) curve analysis was used to assess the discrimination ability of individual metabolites. To assess the ability to discriminate HCC-C from HCC-B using multiple metabolites, we developed a MLR model, for which metabolites were selected by the forward and backward feature selection method using a threshold of $P < 0.2$ for addition and one of $P > 0.2$ for elimination of metabolites. To evaluate the generalization abilities of the developed model, we conducted cross-validation (CV) and bootstrap analysis. In this study, we repeated 200 runs with different random values for each of the 2-fold, 5-fold, and 10-fold CV analyses. Bootstrap

Table 1 Clinical characteristics of the study subjects

	Hepatitis B-associated HCC (n = 30)	Hepatitis C-associated HCC (n = 38)	P value
Sex (M/F)	28/2	22/16	< 0.01 ¹
Age (yr)	62.6 ± 10.9	73.4 ± 9.7	< 0.01 ²
Albumin (g/dL)	3.4 ± 0.6	3.4 ± 0.5	NS ²
Total bilirubin (mg/dL)	1.2 ± 0.6	1.4 ± 1.3	NS ²
AST (U/L)	75.6 ± 76.2	57.8 ± 31.6	NS ²
ALT (U/L)	54.3 ± 38.4	49.8 ± 38.3	NS ²
Fasting blood glucose (mg/dL)	104.5 ± 22.2	113.1 ± 36.0	NS ²
γ-glutamyl transferase (U/L)	77.0 ± 64.8	60.4 ± 58.1	NS ²
Creatinine (mg/dL)	0.73 ± 0.19	1.01 ± 1.53	NS ²
Platelet count (× 10 ³ /μL)	13.5 ± 8.4	11.7 ± 4.7	NS ²
Fib-4 index	5.7 ± 4.4	6.4 ± 3.9	NS ²
AFP (ng/mL) ≤ 6.2 / > 6.2	10/20	8/30	NS ¹
DCP (mAU/mL) ≤ 40 / > 40	17/13	24/14	NS ¹
HBs antigen, positive	30	0	
HCV-antibody, positive	0	38	
HBV genotype (A/B/C/D)	0/14/15/1	NA	
HCV genotype (1b/2a/2b)	NA	34/4/0	

¹Fisher exact test; ²Mann-Whitney U-test. Data were expressed as mean ± SD. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; NS: Not significant; NA: Not applicable; DCP: Des-γ-carboxy prothrombin; HCC: Hepatocellular carcinoma.

analysis was conducted to obtain unbiased estimates of the developed model; 200 repetitions were generated *via* random selection of individuals allowing redundancy. We used JMP (version 10.0.2, SAS Institute, Cary, NC) for the development of the MLR, Weka (version 3.6.10, The University of Waikato, Hamilton, New Zealand) for CV and bootstrap analysis, Mev TM4 software (version 4.7.4, Dana-Farber Cancer Institute, Boston, MA) for clustering and heat map visualization, and GraphPad Prism (version 5.0.4, Intuitive Software, San Diego, CA) for ROC curve analysis and box-plot visualization.

RESULTS

Clinical characteristics of the patients

The characteristics of the HCC patients in this study are shown in Table 1. Thirty and thirty-eight of them had chronic HBV and HCV infection, respectively. Co-infection of HBV and HCV was not found in any of the subjects. Eighteen and 41 patients had AFP (6.2 ng/mL) and des-γ-carboxy prothrombin (DCP) (40 mAU/mL) levels lower than the reference value, respectively. The viral genotype was determined in all cases: genotype B in 14, genotype C in 15 and genotype D in 1 for the HBV genotype, and genotype 1b in 34 and genotype 2a in 4 for the HCV genotype. Higher proportions of men and younger patients were found in the HCC-B group than in the HCC-C group. There were no significant differences in the levels of serum albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, fasting blood glucose, γ-glutamyl transferase, creatinine, platelet count, Fib-4 index, elevated AFP or DCP, between the two groups.

Serum metabolite profiles in virus-related HCC

The serum metabolite profiles of patients with HCC-B or HCC-C are shown as a heat map in Figure 1. Two major patterns of metabolite profiles were identified in cases of virus-related HCC. Cluster X on the horizontal axis of Figure 1 was a characteristic of only HCC-B cases. The other one, shown as cluster Y on the horizontal axis of Figure 1, was characteristic of HCC-C cases and some HCC-B cases. The concentrations of metabolites showed an almost inverse association between these two patterns. γ-Glutamyl peptides were identified as a major marker of HCC. The concentrations of nine γ-glutamyl dipeptides (γ-Glu-Trp, γ-Glu-Tyr, γ-Glu-Phe, γ-Glu-Val, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-His, γ-Glu-Thr and γ-Glu-Glu), as well as those of their binding amino acids (Asp, Glu, Ile, Val, Leu, Thr, Ser, Gly and Ala), were greater in cluster X than in cluster Y, and are shown as cluster A on the vertical axis of Figure 1. On the other hand, the concentrations of four γ-glutamyl peptides, including two γ-glutamyl dipeptides and two γ-glutamyl tripeptides (γ-Glu-Gly-Gly, γ-Glu-Gln, γ-Glu-Asp-Gly and γ-Glu-Met), as well as those of their binding peptides and amino acids (Gly-Gly, Gln and Met), were greater in cluster Y than in cluster X, and are shown as cluster B on the vertical axis of Figure 1. In addition to the γ-glutamyl peptides, 1-methyladenosine, which is a modified nucleoside induced by the post-transcriptional methylation of adenosine by methyl-1-adenosine transferase, was also demonstrated to be a potential marker of HCC. The usefulness of 1-methyladenosine for a diagnostic marker of HCC was first reported by Chen *et al.*^[39], the current study replicated this importance and demonstrated that the concentration of 1-methyladenosine was increased in cluster Y.

Comparison of serum metabolite levels between HCC-B and HCC-C

The serum levels of metabolites were compared between HCC-B and HCC-C. ROC curve analysis was conducted to calculate the area under the curve (AUC) values for all metabolites. The AUC values for individual metabolites showing significant ($P < 0.05$) ability to discriminate between HCC-B and HCC-C are visualized in Figure 2. These metabolites included 15 γ-glutamyl peptides (12 γ-glutamyl dipeptides and 3 γ-glutamyl tripeptides) and 12 amino acids. The 23 metabolites showed relatively high AUC values of > 0.75, and glutamic acid exhibited the highest value at 0.89 (95%CI: 0.79-0.98, $P < 0.0001$). Of these metabolites, methionine, γ-Glu-Gly-Gly and glutamic acid were selected for the MLR model using the feature selection procedure (Table 2). Serum concentrations of glutamic acid were significantly lower in HCC-C than in HCC-B, whereas those of methionine and γ-Glu-Gly-Gly were significantly higher in HCC-C than in HCC-B (glutamic acid: 475.4 ± 206.7 vs 182.6 ± 71.7, $P < 0.0001$; methionine: 37.1 ± 36.1 vs 52.6 ± 29.2, $P =$

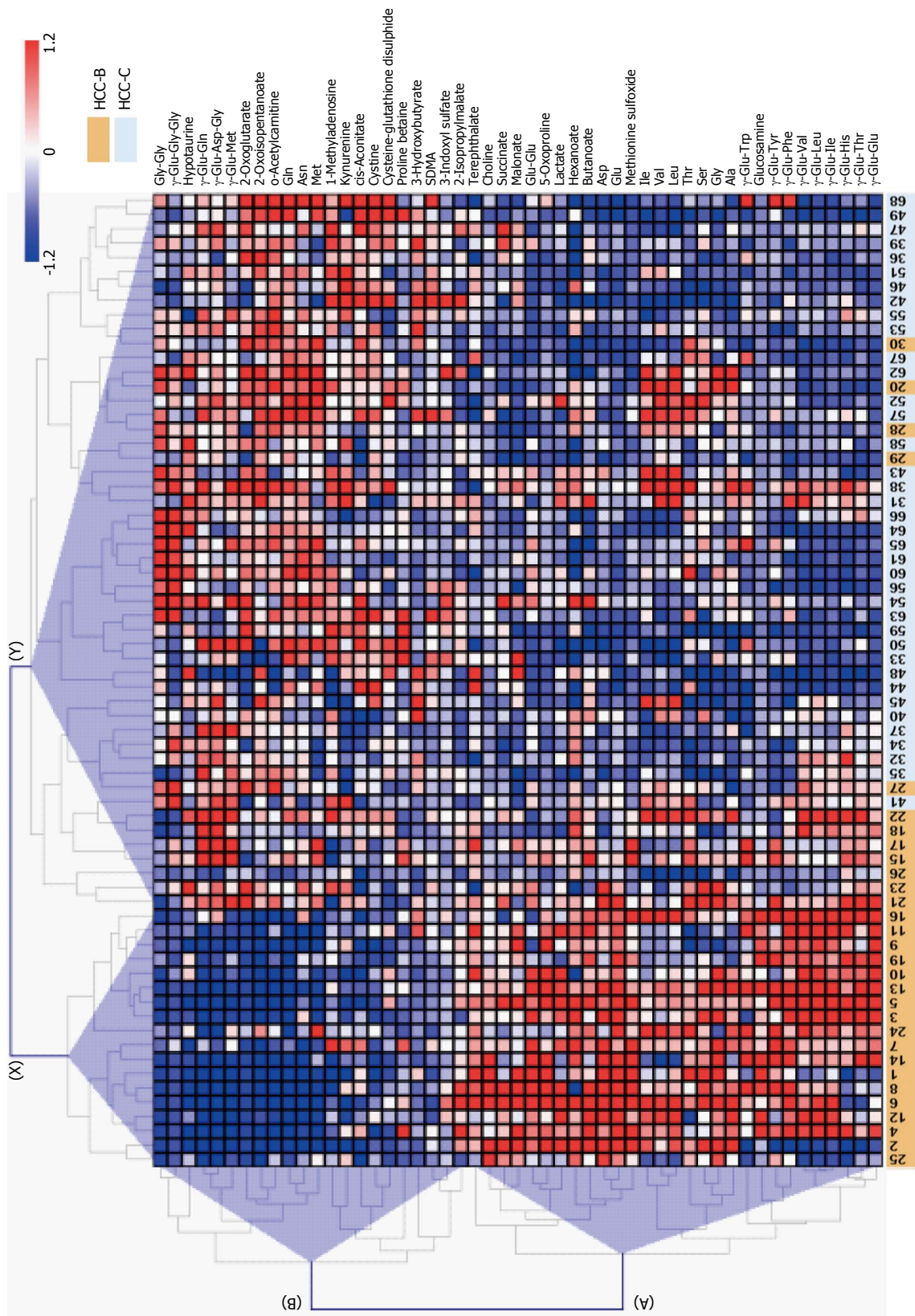


Figure 1 Heat map of quantified metabolites showing significant differences ($P < 0.05$; Mann-Whitney test) between the hepatitis B virus-related hepatocellular carcinoma and hepatitis C virus-related hepatocellular carcinoma groups. The colors on the heat map were determined by the Z-score, reflecting the relative concentrations. The red, blue, and white colors indicate relatively higher, lower and average concentrations, respectively. The numbers at the bottom indicate the case number where orange and light blue indicate HCC-B and HCC-C, respectively. The clusters of metabolites and individuals are labeled A-B on the vertical axis and X-Y on the horizontal axis, respectively. HCC-B: Hepatitis B virus-related HCC; HCC-C: Hepatitis C virus-related HCC; HCC: Hepatocellular carcinoma.

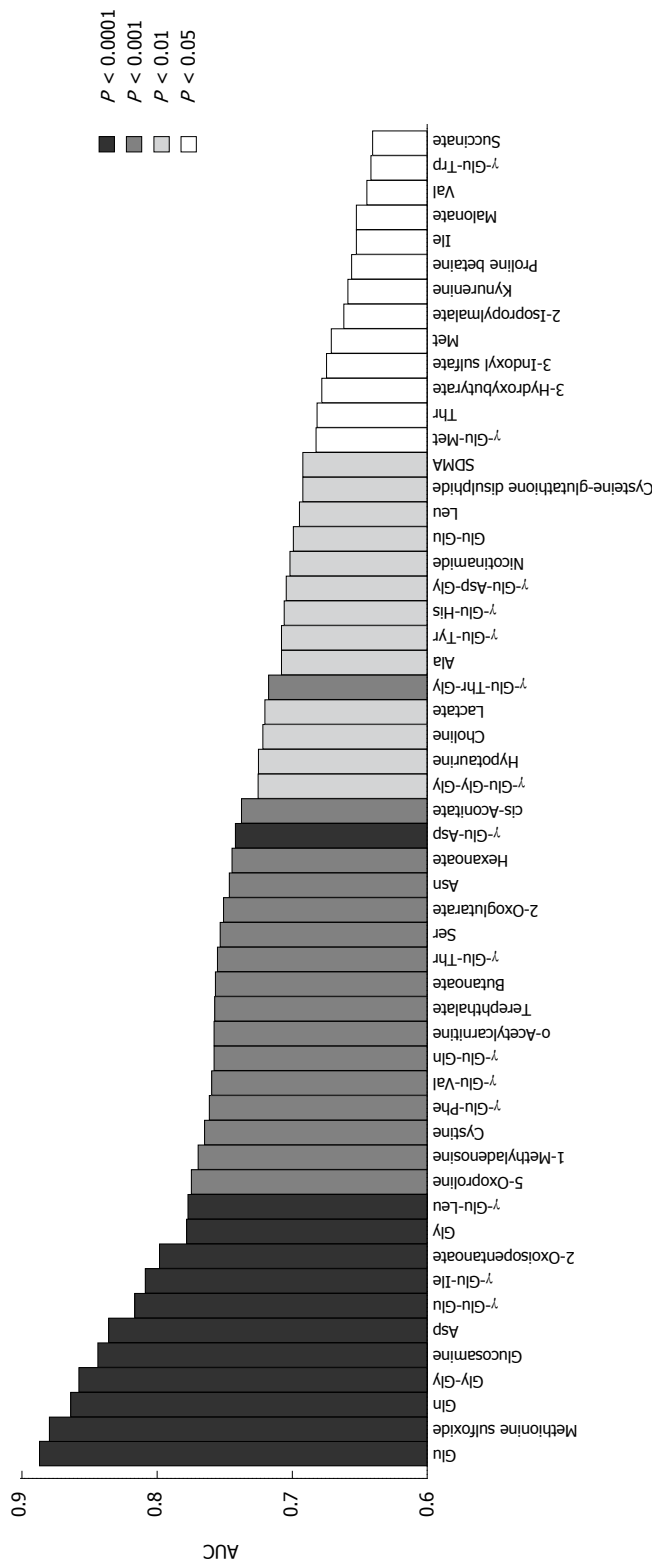


Figure 2 Area under the curve values of individual metabolites showing significant ability to discriminate ($P < 0.05$) between hepatitis B virus-related hepatocellular carcinoma and hepatitis C virus-related hepatocellular carcinoma by receiver operating characteristic analysis. The color corresponds to the level of statistical significance. HCC-B: Hepatitis B virus-related HCC; HCC-C: Hepatitis C virus-related HCC; HCC: Hepatocellular carcinoma.

0.016; γ -Glu-Gly-Gly: 0.035 ± 0.052 vs 0.109 ± 0.106 , $P = 0.001$, HCC-B vs HCC-C, mean \pm SD) (Figure 3A-C). The MLR model using these three metabolites showed significant ability to discriminate between HCC-B and HCC-C (probability of HCC %: 17.6 ± 31.3 vs 86.1 ± 14.2 , $P < 0.0001$, HCC-B vs HCC-C, mean \pm standard deviation) (Figure 3D). The AUC values of the three metabolites for discriminating HCC-C from HCC-B were 0.89 (95%CI: 0.79-0.98, $P < 0.0001$) for glutamic acid, 0.67 (95%CI: 0.53-0.81, $P = 0.016$) for methionine, and 0.73 (95%CI: 0.61-0.85, $P = 0.001$) for γ -Glu-Gly-Gly (Figure 4A-C). The ROC curve for the MLR model incorporating the levels of these three metabolites for discriminating HCC-C from HCC-B showed a high and significant AUC value (AUC = 0.94, 95%CI: 0.89-1.0, $P < 0.0001$) (Figure 4D). The model also showed high AUC values in each of the 200 repetitions of CV analysis, with a median of 0.91 (0.87-0.93, from minimum to maximum), 0.91 (0.85-0.94), and 0.91 (0.73-0.94), for 10-fold, 5-fold, and 2-fold CV, respectively. Bootstrap analysis also yielded high AUC values of 0.95 (0.86-1.0). The results of both analyses indicated the high generalization ability of the developed model.

Correlation of serum concentrations between γ -glutamyl peptides and their binding amino acids

The serum concentrations of most γ -glutamyl peptides were correlated significantly with those of their binding amino acids (Table 3).

DISCUSSION

The progression of liver disease in patients with chronic HBV or HCV infection, which ultimately leads to HCC, is attributable to long-standing inflammation and fibrosis.

Table 2 Parameters of multiple logistic regression model

Metabolite	Coefficient	95%CI		Odds ratio	95%CI		P value
(Intercept)	-8.01	-14.0	-4.14				1.00×10^{-3}
Met	0.0521	0.018	0.0979	1.05	1.02	1.10	8.60×10^{-3}
γ -Glu-Gly-Gly	-17.6	-35.7	-5.31	2.23×10^{-8}	3.18×10^{-16}	4.93×10^{-3}	1.97×10^{-2}
Glu	0.0255	0.0135	0.0454	1.03	1.01	1.05	1.30×10^{-3}

Met: Methionine; Glu: Glutamic acid; γ -Glu-Gly-Gly: γ -glutamyl-glycine-glycine.

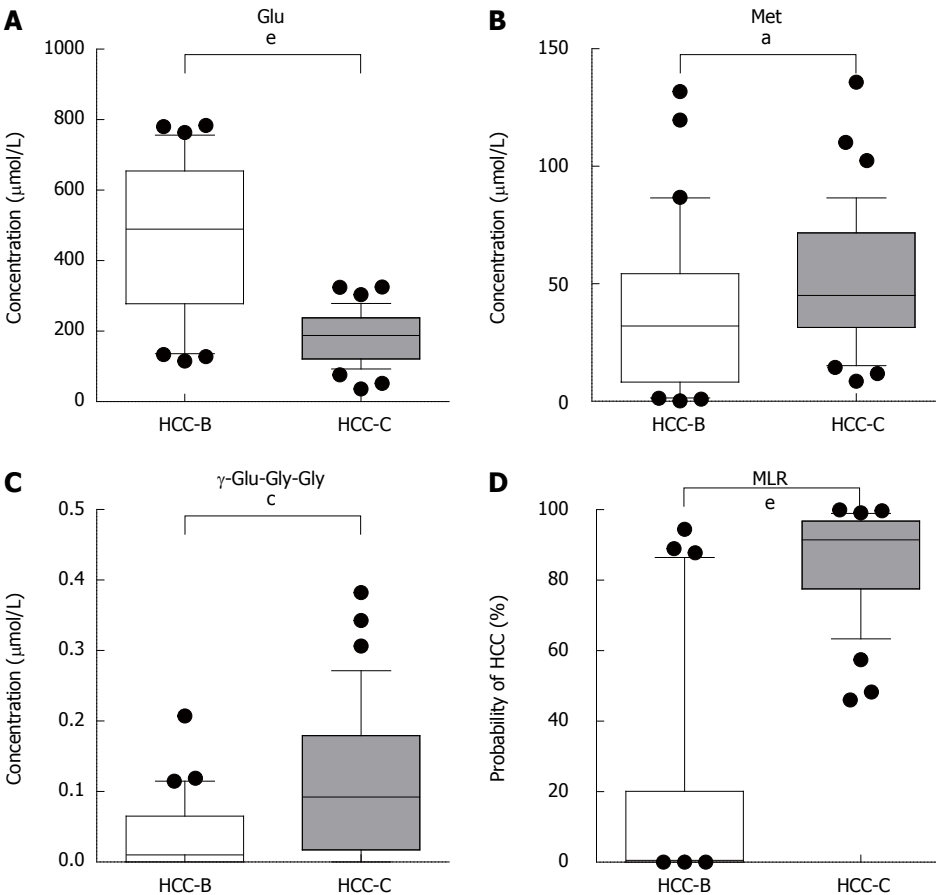


Figure 3 Serum concentrations of glutamic acid (A), methionine (B) and γ -glutamyl-glycine-glycine (C) in hepatitis B virus-related hepatocellular carcinoma and hepatitis C virus-related hepatocellular carcinoma. The multiple logistic regression model using these three metabolites demonstrated that they had a significant ability to discriminate between HCC-B and HCC-C (D). $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.0001$ between groups. Glu: Glutamic acid; Met: Methionine; γ -Glu-Gly-Gly: γ -glutamyl-glycine-glycine; MLR: Multiple logistic regression; HCC-B: Hepatitis B virus-related HCC; HCC-C: Hepatitis C virus-related HCC; HCC: Hepatocellular carcinoma.

As oxidative stress plays an important role in this clinical course^[3-9], the metabolites accompanying production of oxidative stress would be likely candidates for peptide-based biomarker diagnosis of HCC^[26,27]. From this viewpoint, we have focused on the metabolites associated with glutathione metabolism as indicators for monitoring of liver disease, because glutathione metabolism in liver cells plays a significant role in protecting them against ROS^[15-17]. Specifically, the γ -glutamyl peptides are potential candidate biomarkers of liver disease because they are formed by the binding of glutamic acid to various amino acids through catalysis by γ -glutamylcysteine synthetase, and are produced as by-products of glutathione, which confers a protective effect against oxidative

stress^[17,28,29]. The γ -glutamyl cycle is activated by glutathione production in patients with liver diseases such as hepatitis, the glutathione being consumed to neutralize generated ROS, which, in turn, leads to activation of γ -glutamylcysteine synthetase and results in biosynthesis of glutathione together with γ -glutamyl peptides. Our previous metabolome study demonstrated that γ -glutamyl dipeptides can be useful indicators of liver diseases including HCC^[17].

In this study, high concentrations of γ -glutamyl peptides, including 11 γ -glutamyl dipeptides and 2 γ -glutamyl tripeptides, were demonstrated in patients with virus-related HCC. The concentrations of the amino acids that composed these γ -glutamyl peptides were also high, and were significantly correlated

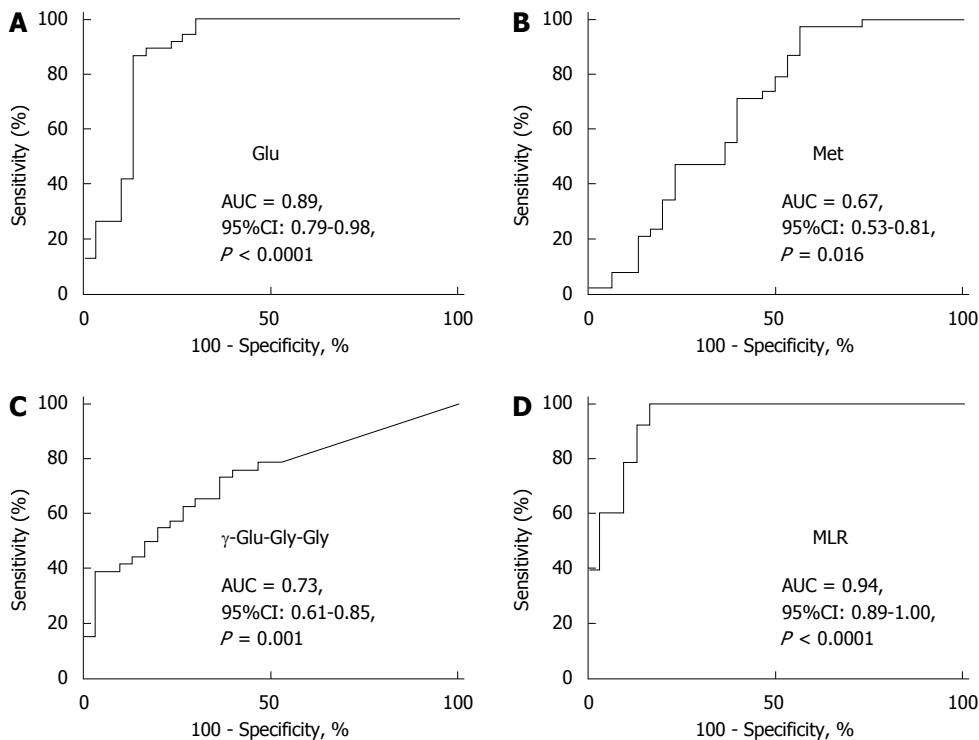


Figure 4 Area under the curve values for glutamic acid (A), methionine (B) and γ -glutamyl-glycine-glycine (C), and the multiple logistic regression model incorporating the concentrations of these three metabolites (D) for discriminating hepatitis B virus-related hepatocellular carcinoma from hepatitis C virus-related hepatocellular carcinoma by receiver operating characteristic analysis. Glu: Glutamic acid; Met: Methionine; γ -Glu-Gly-Gly: γ -glutamyl-glycine-glycine; MLR: Multiple logistic regression; AUC: Area under the curve; HCC-B: Hepatitis B virus-related HCC; HCC-C: Hepatitis C virus-related HCC; HCC: Hepatocellular carcinoma.

Table 3 Correlation coefficients between amino acids and γ -glutamyl peptides

Amino acid	γ -glutamyl peptides	Spearman R	95%CI	P value (two-tailed)
Ile	γ -Glu-Ile	0.4584	0.2401-0.6325	< 0.0001
Ala	γ -Glu-Ala	0.04022	-0.4898	0.7447
Ser	γ -Glu-Ser	0.2117	-0.4698	0.0831
Val	γ -Glu-Val	0.4565	0.2379-0.6311	< 0.0001
Thr	γ -Glu-Thr	0.3909	0.1610-0.5805	0.001
Leu	γ -Glu-Leu	0.4898	0.2779-0.6562	< 0.0001
Asn	γ -Glu-Asn	0.4012	0.1729-0.5885	0.0007
Lys	γ -Glu-Lys	0.2909	0.04915-0.5004	0.0161
Gln	γ -Glu-Gln	0.6387	0.4665-0.7642	< 0.0001
Glu	γ -Glu-Glu	0.8268	0.7294-0.8913	< 0.0001
His	γ -Glu-His	0.2982	0.05714-0.5064	0.0135
Phe	γ -Glu-Phe	0.4886	0.2765-0.6554	< 0.0001
Arg	γ -Glu-Arg	0.4258	0.2016-0.6076	0.0003
Tyr	γ -Glu-Tyr	0.6937	0.5404-0.8024	< 0.0001
Asp	γ -Glu-Asp	0.5566	0.3606-0.7056	< 0.0001
Met	γ -Glu-Met	0.5418	0.3420-0.6947	< 0.0001
Trp	γ -Glu-Trp	0.3433	0.1070-0.5428	0.0042
Gly	γ -Glu-Gly	0.05704	-0.489	0.6441
Gly	γ -Glu-Gly-Gly	0.006012	-0.4905	0.9612

with those of their γ -glutamyl peptides. Metabolome analysis of the present set of HCC cases enabled us to separate them into two metabolite clusters: pattern X representing HCC-B, and pattern Y representing HCC-C and a proportion of HCC-B. Although both patterns included various γ -glutamyl peptides, the concentrations of metabolites were characteristic to

each cluster pattern. In cluster pattern X that included only HCC-B, higher concentrations of metabolites related to glutathione metabolism, γ -glutamyl peptides and their binding amino acids, were notable. In cluster pattern Y including all the HCC-C and some of the HCC-B, higher concentrations of not only metabolites related to glutathione metabolism but also those associated with fatty acid metabolism and the anti-inflammatory response, such as *o*-acetylcarnitine and kynurenine, were evident. These findings suggest that the liver metabolism finally resulting in cancer through long-term viral infection might have differed between the two patterns.

The two major metabolite clusters shown by the heat map indicated that they were roughly divisible into two categories representing HCC-B and HCC-C. In fact, the concentrations of many metabolites differed significantly between HCC-B and HCC-C, and MLR analysis using three chosen metabolites - glutamic acid, methionine and γ -Glu-Gly-Gly - was able to discriminate cases of HCC-C from those of HCC-B with a high degree of significance ($P < 0.0001$). This difference in the profiles of metabolites may reflect the fact that the pathway of cancer occurrence may differ between HCC-B and HCC-C. It is well known that chronic HCV infection is accompanied by metabolic disorders such as lipid metabolic disorder^[30,31] and/or glucose metabolic disorder^[32-34], both of which are closely associated with occurrence of HCC, and such impairment of metabolism has been reported

in the metabolome of HCV-infected cells^[35]. Patients infected with HBV or HCV show impairments of the protective mechanisms against oxidative stress^[3-9]. The life-cycle of the virus in liver cells differs between HBV and HCV^[36]. Such pathological and virological differences between patients with HBV and HCV infection may influence the metabolic pathway that culminates in HCC as an end-stage liver disease, thus possibly resulting in different patterns of metabolite concentrations between HCC-B and HCC-C.

Other than γ -glutamyl peptides, 1-methyladenosine has also been demonstrated to be characteristic of HCC. The concentration of 1-methyladenosine in urine has been reported to be increased in patients with ovarian and breast cancer^[37,38]. Chen *et al.*^[39] identified serum 1-methyladenosine as a characteristic metabolite of HCC for the first time by a liquid chromatography-MS-based metabolome study, and reported that elevation of its serum concentration was applicable as an additional diagnostic biomarker for HCC, particularly in combination with serum AFP. The increased serum concentration of 1-methyladenosine in HCC patients may reflect the hyperactive nucleoside modification associated with hyper methylation in response to long-term inflammation and oxidative DNA damage, which have often been found in patients with cancers of other digestive organs^[40,41]. In the present study, 1-methyladenosine clearly discriminated HCC in cluster pattern Y, therefore, it is expected to become an indicator in this type of HCC. As the limitations of this study, many conditions that may affect the concentration and variation of metabolites in patient's serum, such as the stage of tumor, the stage of fibrosis or inflammation in the liver, their received treatment and viral load, were not identical among the cases studied. Further studies are needed to validate the present findings in a larger cohort of patients.

In conclusion, the present study has shown that high concentrations of γ -glutamyl peptides, including 11 γ -glutamyl dipeptides and 2 γ -glutamyl tripeptides, were demonstrated in patients with virus-related HCC. Two major patterns of metabolite profiles identified were roughly divisible into two categories representing HCC-B and HCC-C. These findings contribute to the development of potential biomarkers for virus-related HCC. The difference in metabolite profiles between HCC-B and HCC-C may reflect the respective metabolic reactions that underlie the different pathogenesises of these two types of HCC.

COMMENTS

Background

Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is a major causes of hepatocellular carcinoma (HCC) worldwide. In this study, the authors conducted a metabolome analysis of serum samples of HBV-related HCC (HCC-B) and HCV-related HCC (HCC-C) using capillary electrophoresis chromatography and mass spectrometry, trying to explore a useful biomarker for virus-related HCC in clinical practice, and yield valuable information on differences in metabolite profiles between HCC-B and HCC-C.

Research frontiers

Metabolome analysis has been used to identify novel biomarkers for various liver diseases, based on the use of liquid chromatography and mass spectrometry or nuclear magnetic resonance and mass spectrometry. The presented study have demonstrated that serum levels of γ -glutamyl dipeptides can be useful indicators of liver diseases.

Innovations and breakthroughs

In this study, high concentrations of γ -glutamyl peptides, including 11 γ -glutamyl dipeptides and 2 γ -glutamyl tripeptides, were demonstrated in patients with virus-related HCC. Two major patterns of metabolite profiles identified were roughly divisible into two categories representing HCC-B and HCC-C.

Applications

The findings of this study contribute to the development of potential biomarkers for virus-related HCC, and they may reflect the respective metabolic reactions that underlie the different pathogenesises of these two types of HCC.

Peer-review

Serum metabolome profiles characterized by patients with hepatocellular carcinoma associated with hepatitis B and C. This is an interesting study. These researches tried to investigate the serum metabolome profiles of patients with hepatocellular carcinoma associated with hepatitis B and C.

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Basic Study

Glucose deprivation induces chemoresistance in colorectal cancer cells by increasing ATF4 expression

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Abstract

AIM: To investigate the role of activating transcription factor 4 (ATF4) in glucose deprivation (GD) induced colorectal cancer (CRC) drug resistance and the mechanism involved.

METHODS: Chemosensitivity and apoptosis were measured under the GD condition. Inhibition of ATF4 using short hairpin RNA in CRC cells under the GD condition and in ATF4-overexpressing CRC cells was performed to identify the role of ATF4 in the GD induced chemoresistance. Quantitative real-time RT-PCR and Western blot were used to detect the mRNA and protein expression of drug resistance gene 1 (*MDR1*), respectively.

RESULTS: GD protected CRC cells from drug-induced apoptosis (oxaliplatin and 5-fluorouracil) and induced the expression of ATF4, a key gene of the unfolded protein response. Depletion of ATF4 in CRC cells under

the GD condition can induce apoptosis and drug re-sensitization. Similarly, inhibition of ATF4 in the ATF4-overexpressing CRC cells reintroduced therapeutic sensitivity and apoptosis. In addition, increased MDR1 expression was observed in GD-treated CRC cells.

CONCLUSION: These data indicate that GD promotes chemoresistance in CRC cells through up-regulating ATF4 expression.

Key words: Glucose deprivation; ATF4; Oxaliplatin; 5-Fluorouracil; Chemoresistance

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Core tip: In this work, we demonstrated that glucose deprivation induces chemoresistance in colorectal cancer (CRC) cells through up-regulating ATF4 expression, and ATF4 is an attractive therapeutic target to combat therapeutic resistance in CRC cells.

Hu YL, Yin Y, Liu HY, Feng YY, Bian ZH, Zhou LY, Zhang JW, Fei BJ, Wang YG, Huang ZH. Glucose deprivation induces chemoresistance in colorectal cancer cells by increasing ATF4 expression. *World J Gastroenterol* 2016; 22(27): 6235-6245 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6235.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6235>

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death worldwide^[1]. Chemotherapy is one of the basic treatments for CRC. However, more than half of CRC patients did not respond to conventional chemotherapy due to drug resistance. Multiple factors contribute to the failure of CRC chemotherapy, including multidrug resistance (MDR) and tumor heterogeneity^[2]. Cancer cells with MDR phenotype simultaneously become resistant to multiple drugs with different structures or cellular targets^[3]. The development of MDR is commonly mediated by multiple factors, including accelerated drug efflux, drug activation and inactivation, alterations in the drug target, repair of drug-induced damage, and escape from apoptosis^[4].

Recent data showed that tumor microenvironment plays a key role in tumor MDR^[5]. In the tumor microenvironment, the abnormal development of vasculature results in insufficient blood supply, which is a key reason for the tumor progression and has been associated with glucose deprivation (GD), chronic hypoxia and other nutrient stress. Increasing evidence indicates that GD promotes tumor cell survival and angiogenesis and induces drug resistance by inducing complex signaling pathways, including unfolded

protein response (UPR)^[6-11]. However, the molecular mechanisms by which cancer cells adapt to GD condition and inhibit drug-induced apoptosis remain poorly understood. Recent studies have indicated that the activating transcription factor 4 (ATF4) pathway, a key player in UPR signaling, is important in regulating malignant phenotypes in various types of human cancers, including breast cancer^[12], CRC^[13], and head and neck squamous cell carcinoma^[14]. In cellular adaptation to tumor GD, the GD activates cell survival through PERK-dependent ATF4 expression. In addition, our previous work revealed that GD and amino acid deprivation promote tumor angiogenesis through activating ATF4^[8,9]. Accumulated data strongly suggest that ATF4 is an important gene in regulating tumor survival under stress conditions, but the functional relationships among cell drug resistance, ATF4 and GD in CRC have not been fully elucidated.

In this study, we investigated whether and how GD affects drug resistance and apoptosis in CRC cells, and revealed that GD induces drug resistance and apoptosis inhibition by activating PERK/ATF4 signaling pathway.

MATERIALS AND METHODS

Cell lines

Human CRC cell lines HCT116 and LoVo were obtained from ATCC. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 23.05 mmol/L glucose (Hyclone) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin at 37 °C in a humidified incubator containing 5% CO₂.

GD treatment

To mimic the GD condition of tumor microenvironment, HCT116 and LoVo cells were incubated for 48 h in DMEM containing 1.5 mmol/L glucose and 10% FBS containing about 0.5 mmol/L glucose at 37 °C in a humidified atmosphere containing 5% CO₂.

Assessment of cell proliferation and chemotherapy sensitivity

For the cell proliferation assay, 1000 CRC cells were plated in 96-well plates and incubated for different time periods (24, 48, 72, and 96 h), and then the cell growth was detected with the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's instructions. For the cell chemotherapy sensitivity assay, 2000 LoVo or HCT116 cells were plated in 96-well plates and treated with oxaliplatin (LOHP; range, 0-16 µg/mL) and 5-fluorouracil (5-FU; range, 0-1.6 µg/mL) for 48 h, and cell inhibition was then assessed by the CCK-8 assay.

Hoechst staining

Hoechst Staining was performed according to the

manufacturer's protocol (Beyotime, China). Cells were visualized with a DP70 inverted immunofluorescence microscope (Olympus). Cells with condensed and fragmented nuclei were judged to be apoptotic.

Quantitative real-time RT-PCR

Total RNA was prepared from cultures on day 10, using the RNAiso reagent (TaKaRa, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the HiFiScript cDNA Kit (CWbio, China). Quantitative real-time RT-PCR analysis was performed to detect mRNA expression using UltraSYBR Mixture (CWbio), with β -actin as an internal control. The sequences of primers used in this study were as follows: 5'-CGTGGTCTTGCTTGGG TG-3' and 5'-TGCGGTGCTTTGCTGGAAT-3' for ATF4; 5'-GACCTATTGGGGTGTTCG-3' and 5'-CCTCAGCGGTTTCTTTCAT-3' for Grp78; 5'-ATAGTGATAAAGGTTTCGGT-3' and 5'-ACAGGAGTTCTGGAAGGAG-3' for PERK; 5'-AGTGTGACGTGGACATCCGCAAAG-3' and 5'-ATCCACATCTGCTGGAAGGTGG AC-3' for β -actin.

Western blot

Cells were lysed with RIPA buffer and incubated on ice for 30 min. After centrifugation, protein concentration was measured with BCA Protein Assay Reagent (CWbio). Cell lysates dissolved in sample buffer were separated using SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After blocking with Tris-buffered saline containing Tween-20 containing 5% milk, the membrane was immunoblotted with appropriate primary antibodies, including anti-Grp78 (Santa Cruz, United States), anti-PERK (Cell Signaling, United States), anti-ATF4 (Santa Cruz), anti-MDR1 (Santa Cruz) and anti- β -actin (Abcam, United States), followed by incubation with goat anti-mouse immunoglobulin (Ig) or anti-rabbit Ig conjugated with horseradish peroxidase. After washing, the membrane was developed using Chemiluminescent Substrate (CWbio).

Plasmid and lentivirus production

Green fluorescent protein-expressing lentiviral plasmids expressing short hairpin RNA (shRNA) against human ATF4 (Vehicle-shATF4) were obtained from Open Biosystems (Carlsbad, CA), and ATF4 lentiviral plasmid (Vehicle-ATF4) was constructed as described in our previous work^[8]. The Vehicle, Vehicle-shATF4, Vehicle and Vehicle-ATF4 plasmids were cotransfected into HEK-293T cells along with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G using Lipofectamine 2000 (Invitrogen). Virus particles were harvested 48 h after cotransfection. Then, the particles were individually used to infect HCT116 and LoVo cells. The cells were then harvested 3 d after infection for Western blot and qRT-PCR validation.

Apoptosis detection

LoVo or HCT116 cells were plated in 6-well plates and treated with LOHP (0.1 μ g/mL) and 5-FU (0.05 μ g/mL) for 48 h. The cells were then harvested and subjected to apoptosis analysis using an Annexin V/7-AAD Apoptosis Detection Kit (CWbio).

Statistical analysis

Each experiment was repeated at least three times. The data are presented as the mean \pm SD. Differences between groups were analyzed with Student's *t* test. All statistical analyses were performed using GraphPad Prism 5 software. The significance level was set at 0.05.

RESULTS

GD decreases sensitivity of CRC cells to chemotherapy and inhibits drug-induced apoptosis

To investigate whether the surviving CRC cells under GD could acquire drug resistance, we assessed the potential effect of GD on the sensitivity of CRC cells to LOHP and 5-FU, two of the most commonly used drugs for CRC treatment^[15]. The results revealed that the IC₅₀ values of GD-treated HCT116/LoVo cells were significantly higher than those of their corresponding control cells (Figure 1A and Figure 2), suggesting that GD strongly decreases the sensitivity of CRC cells to LOHP and 5-FU. These data indicate that GD induces a MDR phenotype in CRC cells. Next, to determine whether GD inhibits chemotherapy-induced apoptosis in CRC cells, we used Hoechst staining to investigate the apoptotic rates. After incubation under GD condition for 24 h, CRC cells were treated with LOHP or 5-FU for subsequent 48 h under normal culture conditions. These cells were then subjected to Hoechst staining. The results revealed that the apoptotic rates were much lower in the GD-treated CRC cells than in the control cells (Figure 1B). To confirm the MDR phenotype of the GD-treated CRC cells, we examined the expression levels of multidrug resistance gene 1 (*MDR1*), a major marker of MDR. As shown in Figure 1C and D, both the mRNA and protein expression levels of *MDR1* were increased in the GD-treated CRC cells as compared to the control cells. Taken together, these observations suggest that GD, through inhibiting apoptosis, significantly decreases the sensitivity of CRC cells to chemotherapy.

Grp78/PERK/ATF4 pathway is activated in GD-induced CRC cells

Our previous work showed that GD induces tumor growth and angiogenesis by activating PERK/ATF4 arm of UPR signaling. To investigate the role of PERK/ATF4 pathway in GD-induced MDR in CRC cells, we examined the mRNA and protein expression of UPR markers (Grp78, PERK and ATF4), which are well-known to be induced by stressful microenvironments such as GD and hypoxia^[8,16]. As expected, the

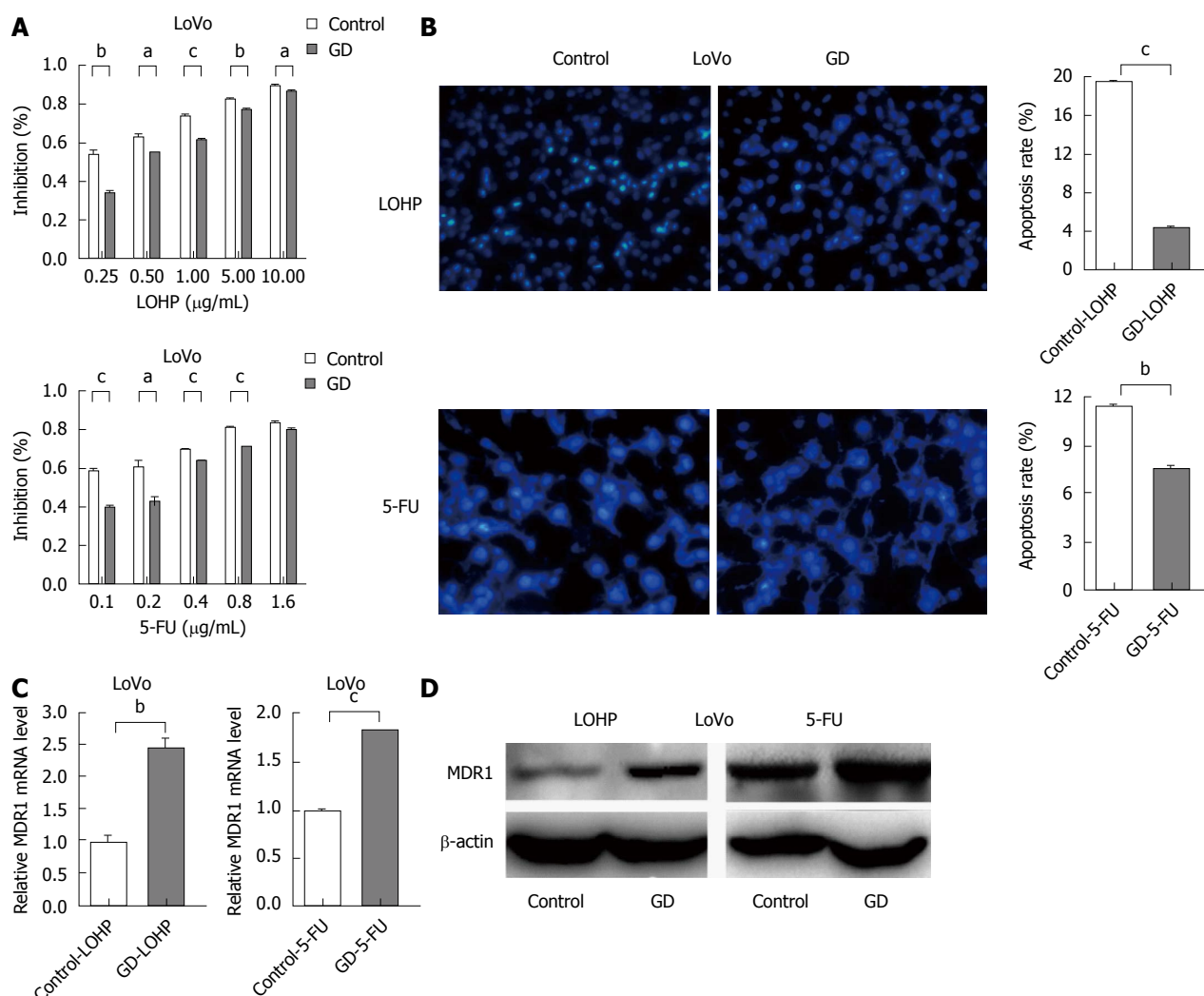


Figure 1 Glucose deprivation promotes drug resistance of colorectal cancer cells. A: GD decreased drug susceptibility to CRC cells. LoVo cells were treated with the indicated doses of the different drugs for 48 h under GD or normal culture condition. The *in vitro* drug sensitivity was tested by the CCK-8 assay; B: GD inhibited LOHP- and 5-FU-induced apoptosis. LoVo cells were treated with 0.1 $\mu\text{g/mL}$ LOHP or 0.05 $\mu\text{g/mL}$ 5-FU for 48 h. Hoechst 33258 nuclear staining and Annexin V/7-AAD staining assays were performed to detect apoptosis. C and D: GD promoted the expression of resistance gene MDR1. The mRNA and protein levels of MDR1 were examined by qRT-PCR and Western blot, respectively. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, control vs GD. GD: Glucose deprivation; CRC: Colorectal cancer.

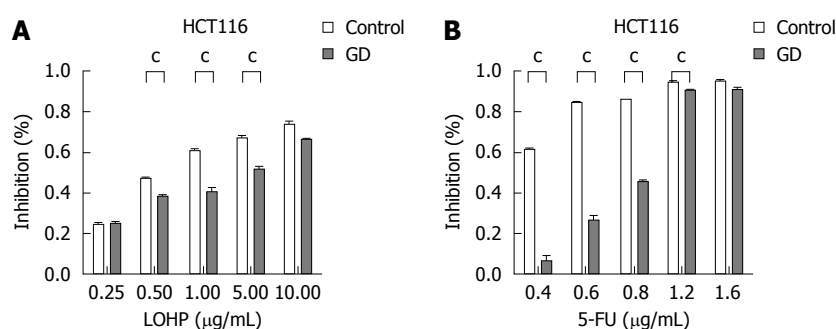


Figure 2 Glucose deprivation promotes drug resistance of HCT116 cells to LOHP and 5-FU. HCT116 cells were treated with the indicated doses of the different drugs for 48 h under GD or the normal condition. The *in vitro* drug sensitivity was tested by the CCK-8 assay. ^c $P < 0.001$, control vs GD. GD: Glucose deprivation.

mRNA levels of Grp78 and ATF4 were significantly increased in GD-treated CRC cells. Although the mRNA and protein expression of PERK was not significantly increased as that of Grp78 and ATF4, the phosphorylation (activation) of PERK (upward shift

in the bands) was clearly observed in GD-treated CRC cells (Figure 3A and B). These data suggest the activation of UPR upon GD treatment and the potential key role of Grp78/PERK/ATF4 pathway in GD-induced MDR phenotype in CRC cells.

ATF4 pathway contributes to GD-induced drug resistance in CRC cells

To explore whether the acquisition of anti-apoptotic property in glucose-depleted CRC cells was due to the activation of ATF4, we silenced the expression of ATF4 using shATF4 in the GD-treated LoVo and HCT116 cells (Figure 4A). The results showed that silencing ATF4 expression counteracted GD-induced drug resistance of CRC cells to both drugs (LOHP and 5-FU) compared with the control cells (Figure 4B and Figure 5A). Moreover, both Hoechst nuclear staining (Figure 5B) and Annexin V/7-AAD staining assays (Figure 5C) showed that ATF4 knockdown significantly increased apoptotic rates of GD-treated CRC cells compared with the control cells. These results suggest that GD inhibits apoptotic activity in CRC cells by activating ATF4 expression. In addition, down-regulation of MDR1 was observed in the ATF4-depleted CRC cells treated with LOHP compared with the control cells, suggesting that ATF4 may mediate GD-induced MDR effect in CRC cells by up-regulating MDR1 expression (Figure 5D). Collectively, these results suggest that the activation of ATF4 plays a crucial role in the GD-induced MDR phenotype in CRC cells.

ATF4 knockdown increases the sensitivity of CRC cells to chemotherapy and counteracts drug-induced apoptosis

To further investigate the role of ATF4 in the drug resistance of CRC cells, forced expression of ATF4 was induced in LoVo and HCT116 cells (LoVo-ATF4 and HCT116-ATF4) using lentivirus transduction. ATF4-overexpressing CRC cells were co-treated with shATF4 and therapeutic drugs, and the results demonstrated that inhibition of ATF4 increased the sensitivity of LoVo-ATF4 and HCT116-ATF4 cells to chemotherapy (Figure 6A and Figure 7A). Moreover, we detected the apoptosis in ATF4-overexpressing cells treated with shATF4, and revealed that the apoptotic rates were much higher compared with the control cells (Figure 6B and Figure 7B). Meanwhile, qRT-PCR and Western blot results also showed the decreased expression of MDR1 (Figure 6C and D). These findings further demonstrate that ATF4 contributes to the induction of chemoresistance in CRC cells.

ATF4 promotes proliferation of CRC cells

Previous studies have proved the role of ATF4 in tumor proliferation. To investigate the proliferation-promoting function of ATF4 in CRC, we overexpressed ATF4 in LoVo and HCT116 cells and then inhibited ATF4 in these cells or their control cells, respectively (As shown in Figure 8A). ATF4 overexpression significantly increased the growth rates of HCT116 and LoVo cells compared to the vector control (Figure 8B). In contrast, inhibition of ATF4 in the ATF4-overexpressing CRC cells significantly decreased the growth rates compared to the control cells (Figure 8C). The results

suggest that ATF4 may play multiple roles in CRC progression.

DISCUSSION

Therapeutic resistance remains a major cause of tumor chemotherapy failure. Its mechanisms are very complicated. Recently, GD has been reported to promote cell proliferation, migration, invasion, angiogenesis and drug resistance in a variety of human cancers through different mechanisms, suggesting its extensive function in tumor development and progression^[6-10,17,18]. UPR is an important mechanism by which GD regulates malignant phenotypes of tumor cells. Our previous work showed that GD contributes to tumor angiogenesis by increasing expression of multiple proangiogenic factors through the PERK/ATF4 signaling, a key signaling pathway in UPR^[8]. In this study, we revealed that GD can decrease the sensitivity of CRC cells to two most commonly used chemotherapeutic drugs (LOHP and 5-FU) in CRC cells by activating PERK/ATF4 pathway. Further analysis showed that silencing ATF4 expression could counteract the inhibitory effect of GD on drug-induced apoptosis, suggesting the key role of ATF4 in GD-induced chemoresistance in CRC.

Due to their rapid and uncontrollable growth, tumors are frequently exposed to extracellular environments that are deficient in nutrients and oxygen, resulting in the disruption of homeostasis in the endoplasmic reticulum (ER) and leading to the activation of UPR. UPR serves to decrease the detrimental effects of accumulated unfolded proteins by increasing protein degradation and decreasing protein synthesis. However, UPR can induce apoptosis in normal cells encountering prolonged stress conditions. Accumulating evidence indicates that UPR contributes to the cancer development, affecting angiogenesis, cell growth, cell differentiation, cell migration, and the inflammatory microenvironment. In addition, recent studies also show that UPR activation can alter the sensitivity of tumor cells to a variety of chemotherapeutic agents. As a common stressful microenvironment in tumor, GD can regulate a variety of tumor phenotypes, mainly by activating UPR pathway. In this study, we revealed that GD induced MDR phenotype by inhibiting 5-FU/LOHP-induced apoptosis in CRC cells. A recent study also reported that COLO-320 colon cancer cells adapted to GD could acquire resistance to doxorubicin-induced apoptosis^[10]. These data demonstrate the key role of GD microenvironment in regulating the MDR phenotype of CRC.

To elucidate the mechanism by which GD induces MDR phenotype in CRC cell, we checked the PERK/ATF4 arm of UPR pathway and revealed its activation under GD condition. In view of the potential role of ATF4 in the UPR and drug resistance in cancer cells,

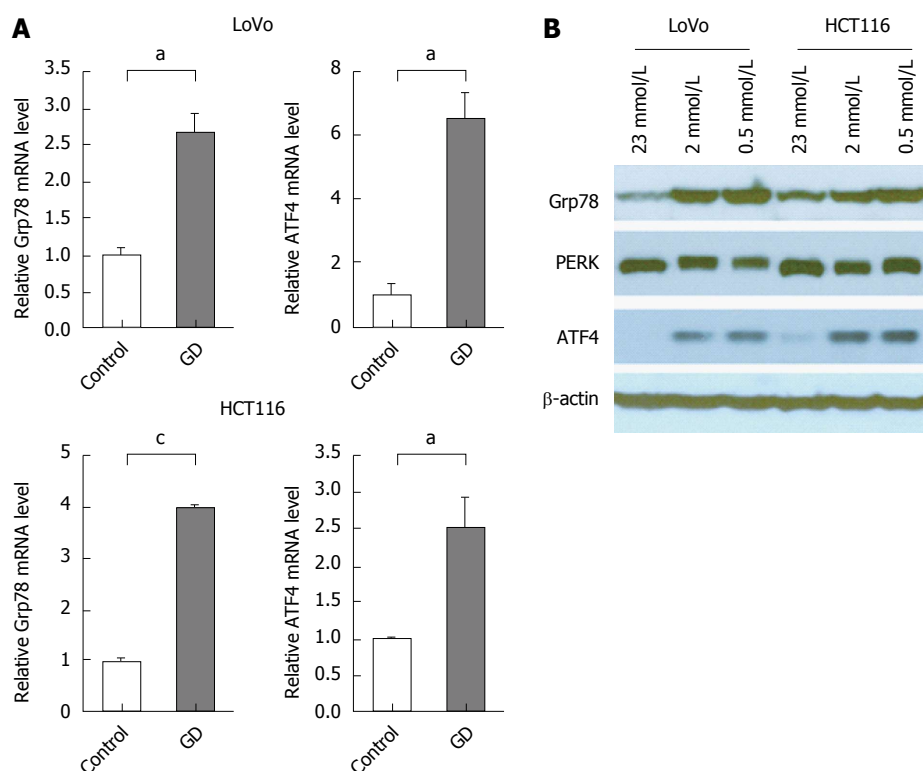


Figure 3 Grp78/PERK/ATF4 pathway is activated in glucose deprivation. A and B: GD promoted the expression of genes involved in UPR. The mRNA and protein expression were examined by qRT-PCR and Western blot, respectively, and β -actin was used as an internal control. The phosphorylation of PERK (upward shift in the bands) indicated its activation in GD-treated CRC cells. ^a $P < 0.05$, ^c $P < 0.001$, control vs GD. GD: Glucose deprivation; CRC: Colorectal cancer.

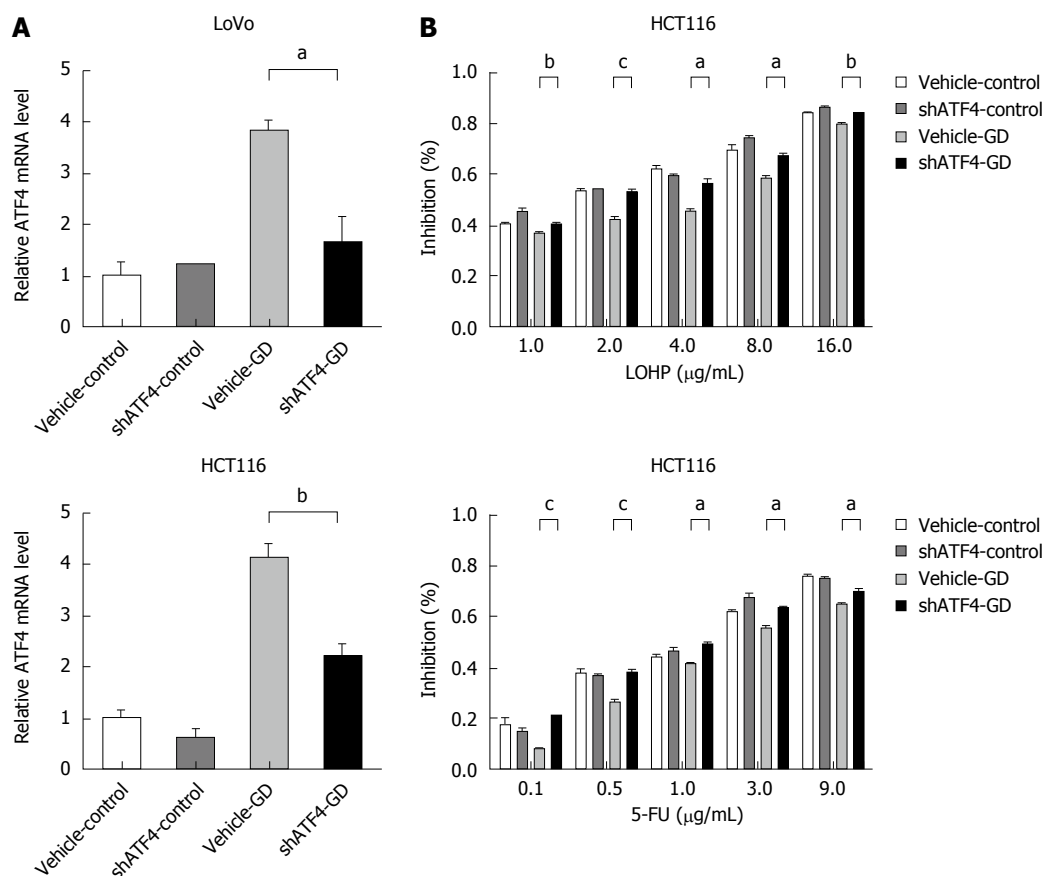


Figure 4 Down-regulation of activating transcription factor 4 significantly reverses the glucose deprivation-induced resistance of HCT116 cells to chemotherapy. A: Silencing ATF4 expression using shATF4 in GD-treated LoVo and HCT116 cells; B: Depletion of ATF4 enhanced the sensitivity of HCT116 cells to LOHP and 5-FU. The *in vitro* drug sensitivity was tested using the CCK-8 assay. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, control vs GD. GD: Glucose deprivation.

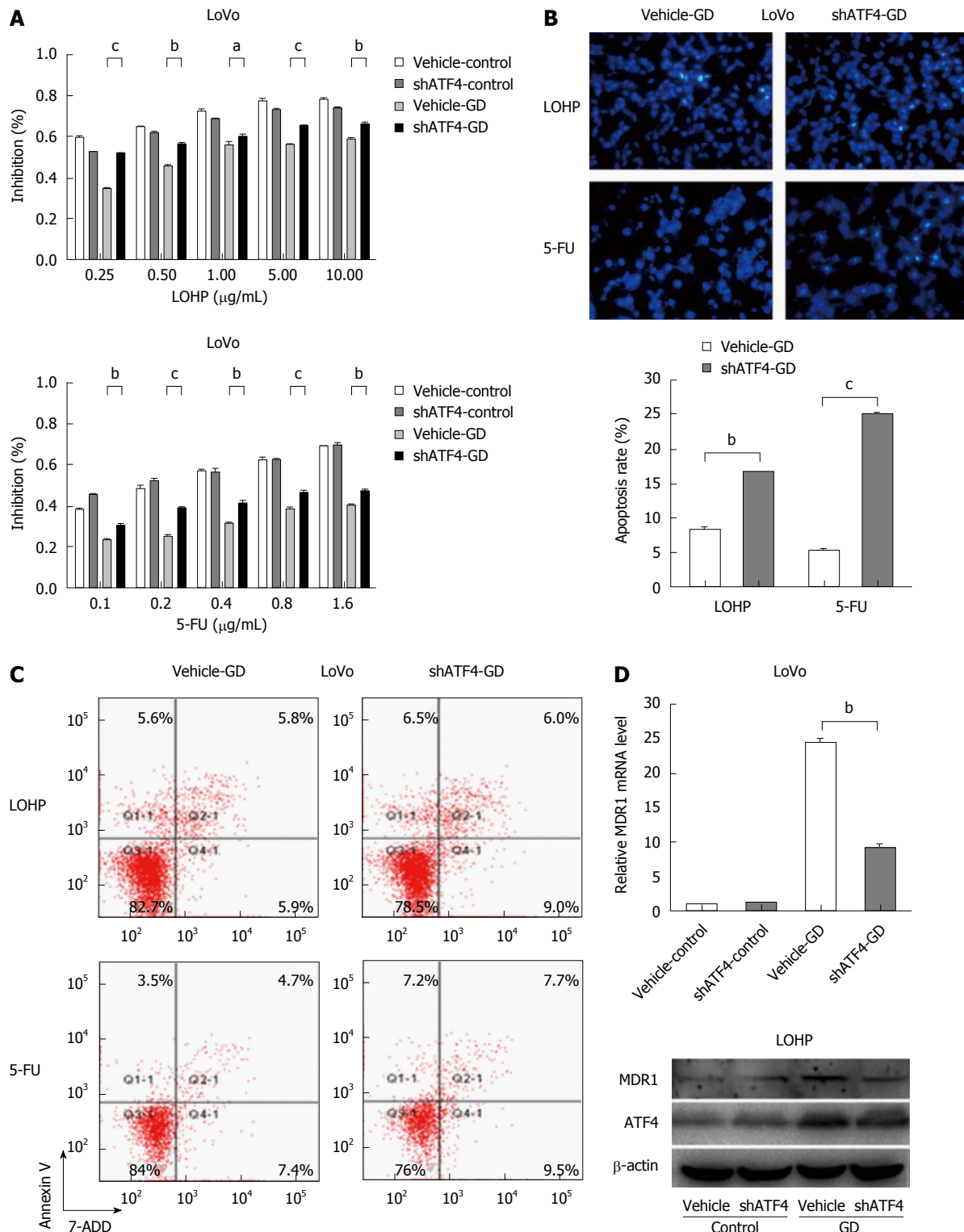


Figure 5 Down-regulation of activating transcription factor 4 significantly reverses the glucose deprivation-induced resistance of colorectal cancer cells to chemotherapy. A: Depletion of ATF4 enhanced the sensitivity of CRC cells to chemical drugs. Vector and shATF4 stably transfected LoVo cells were treated with the indicated doses of the different drugs for 48 h. *In vitro* drug sensitivity was tested using the CCK-8 assay; B and C: The apoptotic rates were much higher in the ATF4-depleted cells than in the control cells. Hoechst 33258 nuclear staining and Annexin V/7-AAD staining assays were performed to detect apoptosis; D: Depletion of ATF4 by shRNA in CRC cells led to significantly reduced expression of MDR1. The mRNA and protein levels of MDR1 were detected by qRT-PCR and Western blot, respectively, and β-actin was used as an internal controls. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001, vehicle GD vs shATF4-GD. GD: Glucose deprivation; CRC: Colorectal cancer.

we tried to reveal its potential influence on the MDR phenotype inducing by GD in CRC cells. As a member of the CREB protein family, ATF4 participates in many intracellular physiological and biochemical processes

and has been suggested as an important target of cancer therapy^[19-26]. For example, ATF4 is the main transcriptional regulator of the cellular hypoxic response to UPR signaling and activates genes that

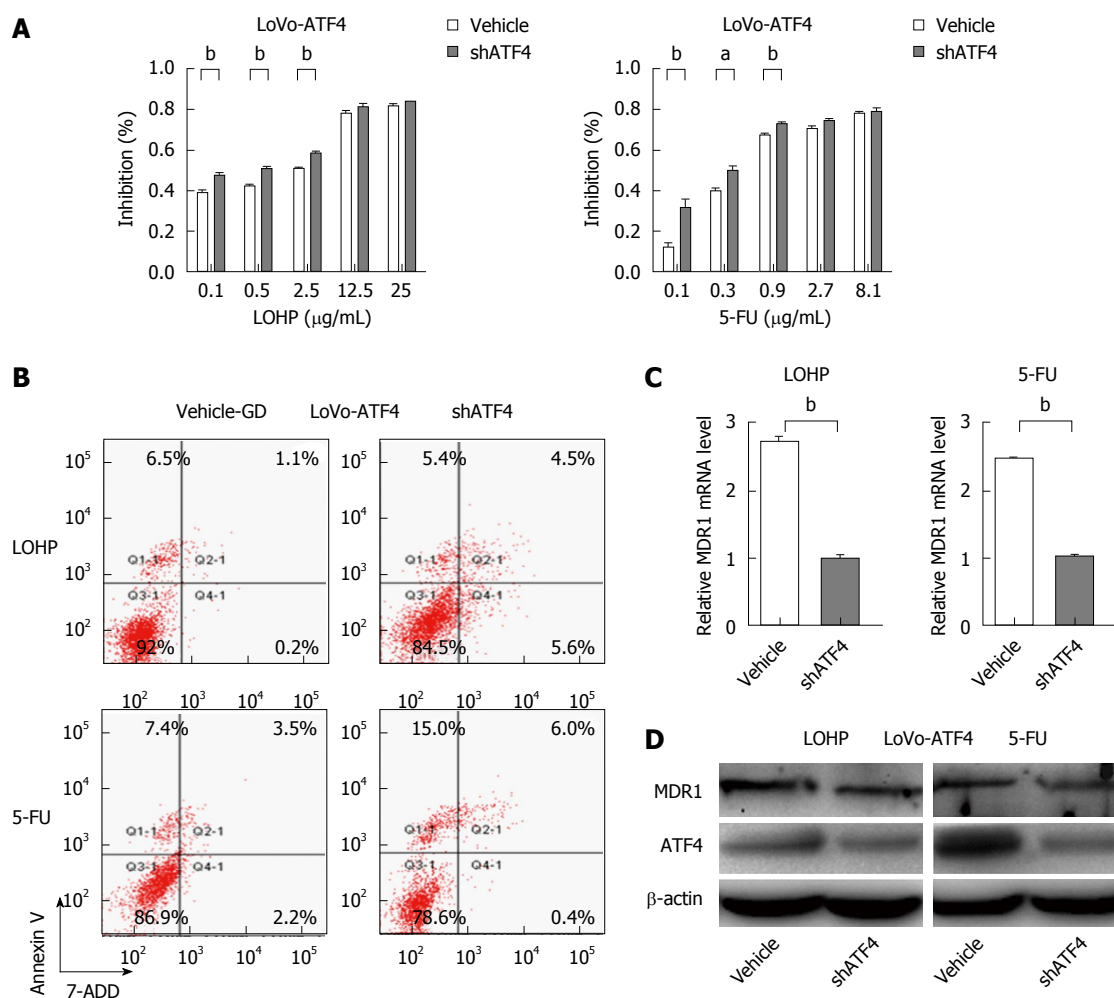


Figure 6 Inhibition of activating transcription factor 4 activity reintroduces drug sensitivity in activating transcription factor 4-overexpressing colorectal cancer cells. A: After transfection with shATF4 or vector, ATF4-overexpressing cells were exposed to the indicated doses of LOHP or 5-FU for 48 h. Cell viabilities were determined by the CCK-8 assay; B: The apoptotic rates were much higher in the shATF4-transfected cells than in the control cells. Annexin V/7-AAD staining assay was performed to detect apoptosis; C and D: Inhibition of ATF4 decreased the MDR1 expression. The mRNA and protein levels of MDR1 in the ATF4-depleted cells were examined by qRT-PCR and Western blot, respectively, and β-actin was used as an internal controls. ^a*P* < 0.05, ^b*P* < 0.01, vehicle vs shATF4. GD: Glucose deprivation; CRC: Colorectal cancer.

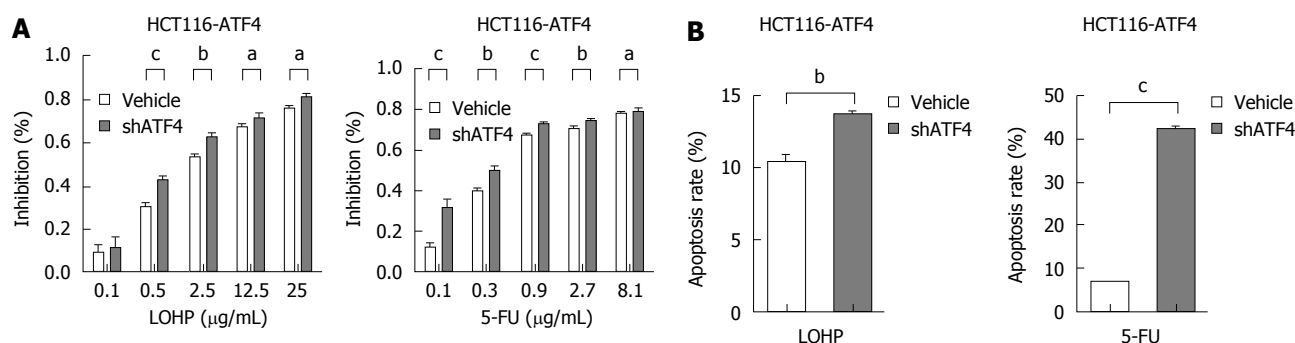


Figure 7 Inhibition of activating transcription factor 4 activity reintroduces drug sensitivity in activating transcription factor 4-overexpressing HCT116 cells. A: ATF4 knockdown increased sensitivity of HCT116-ATF4 cell to chemotherapy. The *in vitro* drug sensitivity was tested using the CCK-8 assay; B: ATF4 knockdown counteracted drug-induced apoptosis. The apoptotic rates were much higher in the shATF4-treated HCT116-ATF4 cells than in the control cells. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001, vehicle vs shATF4.

promote restoration of normal ER function and survival under hypoxia condition^[23]. Recently, ATF4 was reported to promote drug resistance in several types of tumors, including breast cancer^[27], lung cancer^[28], liver cancer^[29], and gastric cancer^[3]. Similarly, we revealed

that the MDR was reversed when we inhibited the GD-induced up-regulation of ATF4 using shATF4. Moreover, we revealed that inhibition of ATF4 by shRNA in the ATF4-overexpressing CRC cells also reintroduced therapeutic sensitivity and apoptosis in CRC cells.

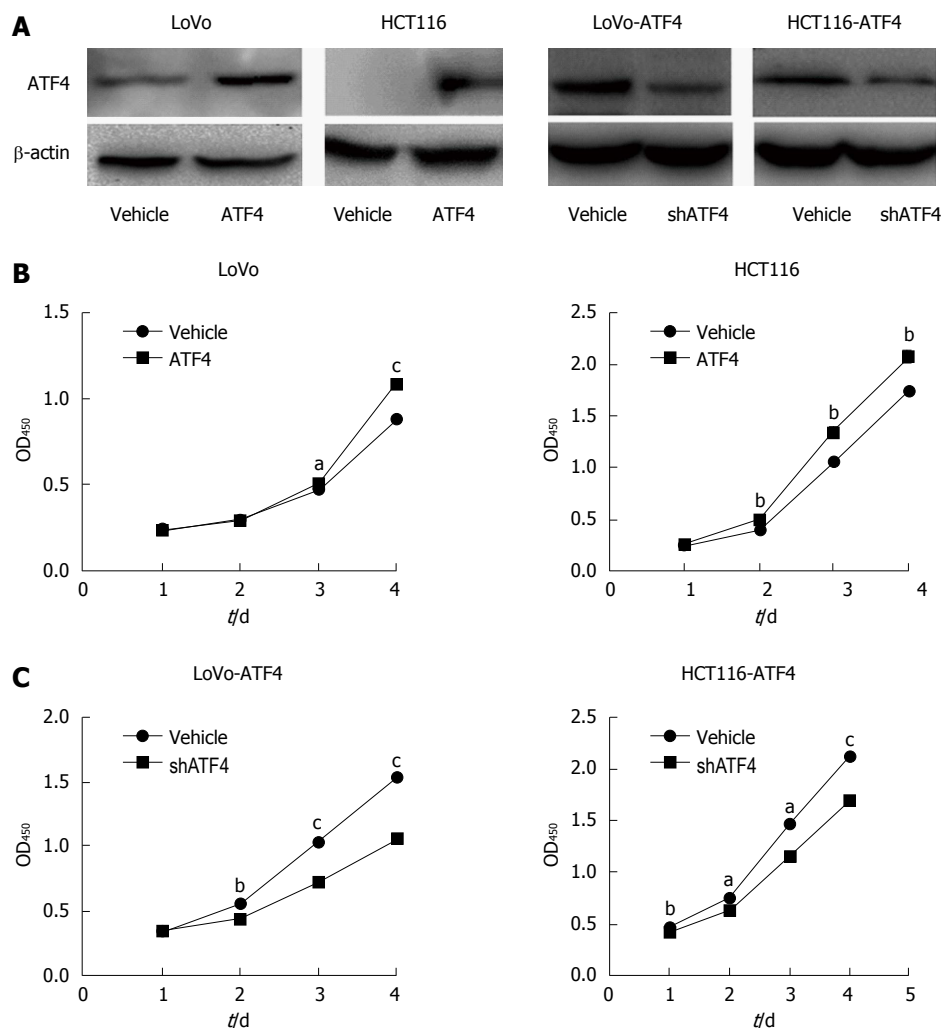


Figure 8 Activating transcription factor 4 promotes the proliferation of colorectal cancer cells. A: We overexpressed ATF4 in LoVo and HCT116 cells and then inhibited ATF4 in these cells or their control cells, respectively; B: ATF4 overexpression enhanced the cell growth of LoVo and HCT116 cells; C: Silencing ATF4 expression inhibited the proliferation of ATF4-overexpressing cells in a dose- and time-dependent manner in LoVo and HCT116 cells. The CCK-8 assay was used to determine the cell growth rate. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, control vs ATF4. GD: Glucose deprivation; CRC: Colorectal cancer.

These data indicate that GD induces MDR mainly by activating ATF4. Nishimoto *et al.*^[10] showed that GD could induce acquire resistance to doxorubicin-induced apoptosis in CRC cells, suggesting that multiple downstream targets mediate the MDR-inducing function of GD.

Ledoux *et al.*^[30] reported that GD enhances expression of MDR1 through c-Jun activation in hepatoma cells. Our data also observed increased MDR1 expression in GD-treated CRC cells. In addition, our data indicated that ATF4 knockdown significantly decreased MDR1 expression in the GD-treated CRC cells compared with the control cells. These results imply that ATF4 contribute to chemoresistance in CRC partly *via* regulating MDR1.

In conclusion, our data clearly identify that GD induces the MDR phenotype of CRC cells by activating PEKR/ATF4 signaling, and targeting the ATF4 pathway may provide a clinical perspective for treating drug resistance of CRC cells to conventional therapy. Further studies are necessary to identify key molecules that

enhance the effect of ATF4 knockdown on CRC cells resistant to conventional chemotherapy. Therefore, interventions based on the disruption of GD-induced ATF4 expression may be effective in reversing drug resistance in CRC cells.

COMMENTS

Background

Chemoresistance is an important reason for clinical chemotherapy failure. Recent studies suggest that tumor microenvironment is an important determinant of malignant progression and chemoresistance. Changes in the tumor microenvironment, such as hypoxia and glucose deprivation (GD), can prompt tumor progression and drug resistance. However, the role and mechanism of GD in colorectal cancer (CRC) drug resistance are unknown.

Research frontiers

GD has been found to be involved in the regulation of multiple pathological processes that contribute to tumorigenesis and metastasis, such as tumor cell proliferation, multidrug resistance, and autophagy. Activating transcription factor 4 (ATF4) is a key player in UPR signaling. Many studies recently revealed that ATF4 participates in drug resistance of cancer. However, the role of ATF4 in GD-induced CRC drug resistance remains unclear and needs further exploration.

Innovations and breakthroughs

The authors clearly identified that GD induces the MDR phenotype of CRC cells by activating PEKR/ATF4 signaling and targeting the ATF4 pathway may provide a clinical perspective for treating drug resistant of CRC to conventional therapy.

Applications

ATF4 may be a therapeutic target to combat therapeutic resistance in CRC cells.

Terminology

GD: In the tumor microenvironment, the abnormal development of vasculature results in insufficient blood supply, such as hypoxia and GD. Changes in the tumor microenvironment can promote tumor progression and drug resistance.

Peer-review

This is a very interesting study which explored the role and mechanism of GD in the chemoresistance of CRC. The results suggest that ATF4 may be a new target for overcoming CRC resistance to conventional chemotherapy.

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Case Control Study

Immunohistochemistry panel segregates molecular types of hepatocellular carcinoma in Brazilian autopsy cases

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Abstract

AIM: To assess the distribution of proteins coded by genes reported as relevant for the molecular classification of hepatocellular carcinoma (HCC).

METHODS: In this retrospective cross-sectional study, the following clinicopathological data were analyzed in 80 autopsied HCC patients: sex, age, ethnicity, alcohol intake, infection with hepatitis B and/or C virus, infection with human immunodeficiency virus, prior treatment, basic and immediate causes of death, liver weight, presence of cirrhosis, number and size of nodules, gross pattern, histological grade and variants, architectural pattern, invasion of large veins, and presence and location of extrahepatic metastases. The protein products of genes known to be involved in molecular pathogenesis of HCC, including epidermal growth factor receptor (EGFR), MET, keratin 19 (K19), vimentin, beta-catenin, mechanistic target of rapamycin (mTOR), extracellular signaling-related

kinase (ERK)1, ERK2, Ki67, cyclin D1, caspase 3 and p53, were detected by immunohistochemistry on tissue microarrays. The expression levels were scored and statistically assessed for correlation with HCC parameters.

RESULTS: Infection with hepatitis C virus was identified in 49% of the 80 autopsy patients, cirrhosis in 90%, advanced tumors in 95%, and extrahepatic metastases in 38%. Expression of K19, p53 and ERK1 correlated to high-grade lesions. Expression of ERK1, nuclear beta-catenin, cyclin D1 and ERK2 correlated to higher rates of cell proliferation as determined by Ki67. Expression of MET, EGFR (> 0) and caspase 3 correlated with lower histological grades. Expression of EGFR correlated to that of caspase 3, and overexpression of EGFR ($\geq 200/300$) was observed in low-grade tumors more frequently (grades 1 and 2: 67% *vs* grade 3: 27% and grade 4: 30%). Expression of ERK1 was associated with that of K19 and vimentin, whereas expression of ERK2 was associated with that of cyclin D1, MET and membrane beta-catenin. Expression of vimentin was strongly correlated with that of K19.

CONCLUSION: Expression of K19, p53, ERK1, ERK2, vimentin and nuclear beta-catenin was related to higher-grade markers, as opposed to expression/overexpression of EGFR, MET and caspase 3.

Key words: Hepatocellular carcinoma; Epidermal growth factor receptor; Autopsy; Immunohistochemistry; Liver; Classification

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Core tip: This study assessed the immunohistochemistry-detected expression of several protein products of genes known to be involved in the molecular pathogenesis of hepatocellular carcinoma (HCC) in a retrospective autopsy cohort of patients with HCC. The data showed that expression profiles of these markers may be related to different pathways underlying HCC progression and metastasis, and that the Edmondson-Steiner's tumor grade may reflect currently recognized molecular subclasses of HCC. This cross-sectional analysis supports the strategy of translating genomic data into panels of immunohistochemical markers for risk evaluation in HCC and also reinforces the paramount importance of histological grade in this context.

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INTRODUCTION

Hepatocellular carcinoma (HCC) has a high incidence in East Asia and Africa, where the rate of infection with hepatitis B virus (HBV) and intake of aflatoxin are more prevalent. Recent years have seen increasing rates of HCC incidence and mortality in western countries, including Brazil, where cirrhosis-related infection with the hepatitis C virus (HCV) predominates^[1,2].

The recent revival of interest in autopsy studies for advanced neoplasms derives from the unique opportunity a corpse presents to assess morphological and molecular aspects of the progression and dissemination patterns of both primary and metastatic tumors^[3-5].

Gene expression studies have proven useful for grouping HCC according to molecular profiles^[6-8]. A meta-analysis of genetic studies by Hoshida *et al*^[9] defined at least two groups of HCC. Subsequent analysis characterized more aggressive types of HCC (Hoshida's S1/S2 subclasses) by the expression of keratin 19 (K19), p53 mutation, and/or regulation by the MET receptor^[10]; moreover, this high-proliferation HCC group also appeared to be related to a stem-cell phenotype. A less aggressive type of HCC (Hoshida's S3 subclass) retains the hepatocyte-like phenotype and includes the molecular categories featuring chromosome 7 polysomy [wherein the epidermal growth factor receptor (*EGFR*) gene and MET oncogene are located] and CTNNB1-mediated activation of the Wnt pathway^[11,12]. Two other molecular categories feature activation of the interferon pathway and amplification of the VEGFA gene^[13].

In the present study, we assessed the expression of protein products of genes that have been reported as relevant for the molecular classification of HCC using an autopsy cohort of patients with HCC.

MATERIALS AND METHODS

Autopsies and tissue microarrays

Among the total 5836 medical autopsies performed in the Pathology Department of the University of Sao Paulo School of Medicine Hospital between the years of 2003 and 2009, 188 presented primary liver tumors. Excluding tumors that were determined to be cholangiocarcinomas ($n = 65$), combined hepatocellular-cholangiocarcinoma ($n = 1$), epithelioid hemangioendothelioma ($n = 1$) and other malignant non-HCC neoplasms and poorly preserved specimens ($n = 13$), 108 cases were confirmed as HCC. These cases, and all related data, were recorded consecutively in the paper files and electronic database of the Hospital das Clínicas of the University of Sao Paulo School Of Medicine, Brazil's largest academic hospital, and were accessed for study in accordance with the investigative protocols of the hospital's Ethics

Table 1 Antibodies and immunohistochemistry parameters used in this study

Antibody	Manufacturer	Clone	Species	Dilution	Retrieval ¹	Positive control	Staining pattern
EGFR PharmDx	Dako	2-18C9	Mouse	Pre-diluted	Proteinase K	Provided by manufacturer	Membrane
Ki67 Ag	Dako	MIB-1	Mouse	1:400	Standard	Tonsil	Nucleus
Caspase 3	DBS	3C SP03	Mouse	1:400	Standard	Tonsil/stomach	Cytoplasm
ERK1	DBS	Polyclonal	Rabbit	1:100	Standard	Breast carcinoma	Cytoplasm
ERK2	DBS	Polyclonal	Rabbit	1:400	Standard	Breast carcinoma	Cytoplasm
MET	Cell signaling	Polyclonal	Rabbit	1:50	Standard	Breast carcinoma	Cytoplasm
mTOR	Cell signaling	Polyclonal	Rabbit	1:50	Standard	Lymphoid tissue	Cytoplasm
K19	Novocastra	b170	Mouse	1:300	Standard	Cholangio-carcinoma	Cytoplasm
Vimentin	Dako	Vim3B4	Mouse	1:3000	Standard	Kidney	Cytoplasm
p53	Dako	DO7	Mouse	1:100	Standard	Breast carcinoma	Nucleus
Beta-catenin	BD	14Beta-catenin	Mouse	1:800	Standard	Tonsil	Membrane/nucleus
Cyclin D1	Dako	SP4	Rabbit	1:100	Steamer 10 mmol/L TRIS, 1 mmol/L EDTA, pH 9	Tonsil/intestine	Nucleus

¹Antigen retrieval was performed by steam heating for 40 min in 10 mmol/L citrate buffer, pH 6. BD: BD biosciences™; DBS: Diagnostic bioSystems™; IHC: Immunohistochemistry; K19: Keratin 19; mTOR: Mechanistic target of rapamycin.

Committee for Research Project Analysis (CAPPesq). Archived pathological slides were reviewed for each case, and sufficient preserved tumor tissue and clinicopathological data was available for 80 of the 108 HCC cases reviewed.

All corpses had been preserved by routine refrigeration. Standard immunostaining practices were used for detection of vimentin expression. Normal (non-cancerous) tissues were also obtained from the HCC patients for use as internal controls and examined to provide evidence of adequate tissue preservation prior to continuing with further immunohistochemistry procedures. Detailed pathological data were recorded. Clinical and demographic data were retrieved from medical records and autopsy reports. The primary objective of the histological review was to define the major architectural patterns and histological grading (1-4) according to the system set forth by Edmondson and Steiner^[14]. Tumors showing heterogeneous histological grades were classified as the highest grade shown^[15]. HCC nodules < 2 cm were considered incidental. Presence of multiple intrahepatic nodules (≥ 4)-regardless of either massive or diffuse type-prompted analysis of primary HCC and intrahepatic metastases as intrahepatic tumors. Paraffin-embedded tissue blocks of primary HCC, extrahepatic metastases and non-neoplastic liver were selected for use in construction of tissue microarray (TMA) and immunohistochemistry (IHC). Liver fibrosis was classified using a standard 0-4 system, wherein F0 indicated no fibrosis, F1 indicated portal fibrosis without septa, F2 indicated portal fibrosis with few septa, F3 indicated numerous septa without cirrhosis, and F4 indicated cirrhosis.

TMAs

TMAs are produced by extracting the tissue cores from many paraffin-donor blocks and then re-embedding into a single recipient block at defined array coordinates. For this study, two or three 1.0 mm

cores of each original sampled tissue-primary HCC, extrahepatic metastases, and non-neoplastic liver-were selected for TMA. Cores of the primary HCC and extrahepatic metastases samples were used to construct two (duplicate) TMAs each, and the cores of the non-neoplastic samples were used to construct one TMA. For heterogeneous tumors, different areas were cored separately. When more than one primary HCC was present in a single case, all tumors were sampled, but data were computed for the largest one. Available non-neoplastic liver samples were selected as far from the tumor border as possible, usually from a different paraffin block. All available extrahepatic metastases and large vein invasion samples were cored.

IHC and case categorization

Table 1 summarizes the protocols of the IHC procedures and respective antibodies used in this study.

In brief, the slide-mounted sections of the paraffin-embedded tissues were deparaffinized and rehydrated. Antigen retrieval consisted of submerging the slides in 10 mM citrate buffer (pH 6) and steam heating for 40 min. After washing with distilled water, blocking of endogenous peroxidase was carried out by incubating with 6% hydrogen peroxide solution in methanol at room temperature for 10 min and repeating twice. Universal protein blocking was carried out by incubating CASBlock™ solution (Invitrogen, United States) at 37 °C for 10 min. Antigen detection with the primary antibody was carried out by incubating first at 37 °C for 30 min and then at 4 °C for 18 h. With the exception of EGFR PharmDx, all signal amplifications were achieved by application of the Novolink™ polymer system (Novocastra, United States) with incubation at 37 °C for 30 min. The immunoreactive signal was visualized by first incubating with chromogen 3-3'-diaminobenzidine (60 mg/dL in a phosphate buffer pH 7.4) at 37 °C for 5 min, followed by washes with distilled water, counterstaining with

Table 2 Clinicopathological features of 80 hepatocellular carcinoma autopsy cases

Feature	n (%)
Sex	
Male	62 (77.5)
Female	18 (22.5)
Age, yr	
Median	59.5
Range	28-82
With cirrhotic liver	72 (90.0)
With incidental small HCC, < 2.0 cm	4 (5.0)
Etiology	
HCV only	27 (33.7)
HCV + alcohol	9 (11.2)
HCV + HBV	2 (2.5)
HCV + HBV + alcohol	1 (1.3)
Alcohol only	11 (13.8)
HBV only	9 (11.2)
HBV + alcohol	3 (3.8)
Hemochromatosis	2 (2.5)
Cryptogenic	10 (12.5)
Data not available	6 (7.5)
Larger tumor size, cm	
Median	4
Range	0.8-18.0
Non-available/non-sizable cases	22 (27.5)
Number of tumor nodules	
1	24 (33.3)
2	4 (5.6)
3	1 (1.4)
≥ 4	43 (53.8)
Non-available/uncountable cases	8 (10.0)
Gross pattern	
Nodular	31 (38.8)
Massive with satellite lesions	25 (31.3)
Diffuse	8 (10.0)
Non-available/non-sizable cases	11 (13.8)
Histological grade, Edmondson-Steiner grade	
1 + 2	22 (27.5)
3	47 (58.8)
4	11 (13.8)
Predominant histological pattern or variant	
Trabecular	36 (45.0)
Acinar/pseudoacinar	9 (11.2)
Solid/macrotrabecular	24 (30.0)
Mixed	9 (11.2)
Clear cell	2 (2.5)

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

Harris' hematoxylin by incubation at room temperature for 1 min, dehydration in a progressive alcohol series, clearing in xylene, and mounting with Entellan™ (Merck, United States).

Assessment of EGFR was carried out by applying the EGFR PharmDx Kit (Dako, United States) according to the manufacturer's instructions. EGFR membrane expression was classified according to staining intensity, with scoring from 0 to 3+, and according to distribution among the HCC cells (0% to 100%). A score from 0 (no staining) to 300 (100% strong staining) was assigned to each spot by two independent pathologists. For spots where the two pathologists gave differing scores, a consensus score was reached with the two working in

tandem on a two-observing microscope. For each case, a final score was calculated as the arithmetical average of all spots for that sample. The score for metastatic disease was calculated as the arithmetical average of the available metastases. Cases or samples were classified as EGFR-expressing when the score was > 0 and subclassified as "EGFR-overexpressing" when the final score was ≥ 200.

Membrane beta-catenin, mechanistic target of rapamycin (mTOR), MET, caspase 3, vimentin, extra-cellular signaling-related kinase (ERK)1 and ERK2 were analyzed using a scoring system similar to that described for EGFR, with both intensity and distribution of immunostaining being considered to obtain a score between 0 and 300. The cut-off values for expression and overexpression were set at 100 (of 300) and 200 (of 300), respectively, for membrane beta-catenin, mTOR, MET, ERK1 and ERK2. Any expression of vimentin in tumor cells was considered abnormal, with the cut-off being 0 (of 300). For caspase 3, an expression score < 10 (of 300) was considered to indicate a "loss of expression".

Nuclear staining of Ki67, cyclin D1 and p53 was calculated for each spot by cell counting, with the result expressed as a percentage of the labeled cells. Nuclear K19 expression was estimated at 10% intervals of positive cells. Nuclear beta-catenin was semi-quantitatively scored at 0 (no staining), 1+ (weak or focal staining), 2+ (moderate staining) and 3+ (diffuse strong staining).

Statistical analysis

SPSS statistical software, version 15.0 (SPSS Inc., United States) was used for all statistical analyses. All numeric variables were tested by the Kolmogorov-Smirnov goodness-of-fit test to assess normal distribution. Frequency distributions of the clinicopathological data and the immunohistochemical categories were assessed using Fisher's exact or χ^2 tests, with the threshold of significance set at 0.05. If any difference was detected in a set of three variables, pairwise tests were used to detect outliers. Spearman's correlation coefficient was used to assess correlations among scores and other categorical variables.

RESULTS

Demographics and pathological data

The clinicopathological data and organ distribution of metastases are summarized in Tables 2 and 3. Patients with cirrhosis (90.0%) accompanied by clinically-relevant non-incidental HCC predominated in this autopsy series (95.0%). HCV infection was a major factor in 39 patients (48.7%), followed by significant alcohol intake (30.0%) and HBV infection (18.7%). A history of chronic alcohol intake was present for male patients exclusively (42.9%, $P < 0.0001$).

The majority of females with HCC had HCV

Table 3 Dissemination in 80 hepatocellular carcinoma autopsy cases

Metastasis feature	n (%)
Large vein invasion	12 (15.0)
Extrahepatic metastases	30 (37.5)
Extrahepatic metastases sites, cases by site	
Lungs	21 (26.3)
Lymph node	6 (7.5)
Adrenal, bone, spleen, diaphragm, peritoneum	2 (2.5)
Small bowel, bladder, colon, pancreas, pituitaries, thyroid, pleura	1 (1.3)

infection (72.2%), compared to less than one-half of the males (41.9%, $P < 0.05$). Females also had a smaller average tumor size (3.2 ± 2.0 cm) than the males (6.0 ± 4.4 cm, $P < 0.01$).

HCC was detected in 8 patients with non-cirrhotic livers (10%), consisting of 4 without fibrosis (F0), 2 with mild fibrosis (F1), 1 with bridging fibrosis (F2), and 1 classified as non-cirrhotic not otherwise specified.

Tumor sizes of ≥ 6.0 cm were found in 80% of the non-cirrhotic livers and in 30.2% of cirrhotic livers ($P < 0.05$). Non-cirrhotic cases also had a higher proportion of unknown risk factors for HCC (62.5%) than the cirrhotic cases (19.4%, $P = 0.02$).

Eleven of the HCC cases (14%) had undergone chemoembolization treatment prior to death, including 1 patient who received chemoembolization plus ethanol injection, 1 who received systemic chemotherapy in addition, and 1 who died of recurrent HCC after subsequent liver transplantation. Most of the HCC cases in our autopsy cohort (82%) had not received specific oncological treatment, mainly due to the acute presentation of advanced disease.

Four cases of incidental small HCC (5.0%) were detected in the cirrhotic livers, comprised of 1 patient with HCV, 1 with HBV, 1 with HBV + alcohol, and 1 of unknown cause; each cases had one or two nodules of size ranging between 0.8 cm and 1.5 cm.

Multiple intrahepatic tumors were detected in 31.6% (6/19) of cases with grade 2 HCC and in 69.8% (37/53) of the cases with grade 3 or 4 when combined ($P < 0.01$). Accordingly, the proportions of HCC cases classified as nodular, massive and diffuse among the grades 3 and 4 combined group were 67.7%, 88% and 75% respectively.

A trabecular pattern was predominant among the cases in the combined-group of patients with tumors of grades 1 and 2 (61.9%) and in patients with grade 3 tumors (47.8%). A solid pattern was seen in 90.9% of the group of patients with grade 4 HCC.

Extrahepatic metastases were detected in 53.5% of cases of multiple intrahepatic tumors, and in 10.3% of cases with one to three intrahepatic tumors ($P < 0.001$). Consistently, cases with extrahepatic metastases had increased liver weight (2388.3 ± 842.1 g) as compared to cases without extrahepatic

metastases (1501.3 ± 625.5 g, $P < 0.01$).

Immunohistochemistry

The expression patterns and cut-off points used for the different markers in primary HCC, metastases and non-neoplastic liver are summarized in Table 4 and shown in Figures 1 and 2. The discrepancy in numbers of cases presented in each table (Tables 4, 5 and 6) reflects the loss of some spots during TMA processing.

The non-neoplastic liver tissues, mostly representative of cirrhosis, showed higher rates of EGFR overexpression, higher caspase 3 expression ($P = 0.009$), lower p53 expression ($P = 0.002$) and lower rates of Ki67-evidenced cell proliferation ($P < 0.001$) than the primary HCC samples. K19 expression was below the threshold of detection for all non-neoplastic hepatocytes examined. High-level expression of ERK1 ($P = 0.006$) and ERK2 ($P = 0.029$) was only detected in tumor samples, and predominantly in metastases samples. Cyclin D1 was more frequently expressed in metastases samples than in other samples ($P = 0.018$). There was a trend towards increased expression of vimentin and nuclear beta-catenin in tumor samples and metastases when compared to non-neoplastic liver samples. The expression of mTOR, MET, ERK1 and ERK2 was higher in metastases samples than in intrahepatic liver tumor samples, although the difference between the two did not reach statistical significance.

Table 5 summarizes the relation of histological grade and expression of the immunohistochemical markers of HCC. While EGFR overexpression and MET expression were more common in the combined-group of patients with grades 1 and 2 HCC, K19 expression and loss of caspase 3 were more frequent in the group with grade 4 HCC. The combined-group of patients with grades 1 and 2 HCC showed a trend towards very low Ki67 index ($< 0.1\%$).

Table 6 presents the association coefficients for the scores or values of the immunohistochemical markers of HCC and the grade of primary HCC. Although the association coefficients were mostly moderate or weak, some clear segregation existed between markers that were positively associated with the histological grade and cell proliferation (such as p53, K19, and nuclear beta-catenin-in group 1) vs markers with the opposite profile (e.g., MET, EGFR and caspase 3-in group 3). Vimentin was significantly associated with K19. In group 2, the immunohistochemical markers of mTOR, membrane beta-catenin and cyclin D1 showed intermediate characteristics, and were less associated with histological grade, but still showed association with Ki67-evidenced cell proliferation. ERK1 and ERK2 were both associated with Ki67-evidenced cell proliferation and with each other's expression. However, ERK1 was associated with K19 and vimentin, while ERK2 was associated with cyclin D1, MET and membrane beta-catenin.

Table 4 Overall expression and cut-off points for immunohistochemical markers in non-neoplastic liver, primary hepatocellular carcinoma and metastases *n* (%)

IHC marker	Cut-off	Non-neoplastic 26 cases	Primary HCC 75 cases	Metastases 17 cases	<i>P</i> value
EGFR overexpression	$\geq 200/300^1$	17 (65)	29 (39)	6 (38) ²	0.054
Ki67	$\geq 0.1\%$	2 (8) ^a	45 (63) ⁴	13 (76)	< 0.001 ^a
K19	> 1%	0 (0) ^a	12 (16) ³	5 (29)	0.011 ^a
Vimentin	> 0/300	0 (0)	4 (6) ³	3 (18)	0.064
Caspase 3 loss	$\leq 10/300$	2 (8) ^a	26 (36) ³	7 (41)	0.009 ^a
Cyclin D1	> 0.1%	4 (15)	16 (23) ⁵	9 (53) ^a	0.018 ^a
mTOR expression	$\geq 100/300^1$	8 (31)	14 (21) ⁵	5 (31) ²	0.533
MET expression	$\geq 100/300^1$	10 (38)	12 (17) ⁴	5 (29)	0.068
ERK1 overexpression	$\geq 200/300^1$	0 (0) ^a	10 (14) ⁴	5 (33) ³	0.006 ^a
ERK2 overexpression	$\geq 200/300^1$	0 (0) ^a	10 (14) ⁴	4 (24)	0.029 ^a
p53	$\geq 10\%$	2 (8) ^a	28 (42) ⁵	8 (50) ²	0.002 ^a
Beta-catenin, membrane	$\geq 100/300^1$	10 (38)	42 (61) ⁵	11 (65)	0.113
Beta-catenin, nucleus	2 or 3+	0 (0)	9 (9) ⁵	3 (18)	0.070

¹Score value; ²One case lost during technical processing (*n*-1); ³Two cases lost during technical processing (*n*-2); ⁴Three cases lost during technical processing (*n*-3); ⁵Four or more cases lost during technical processing. ^a*P* < 0.05 (outliers in pairwise test). EGFR: Epidermal growth factor receptor; ERK: Extracellular signaling-related kinase; HCC: Hepatocellular carcinoma; IHC: Immunohistochemistry; MET: MET Receptor tyrosine kinase; mTOR: Mechanistic target of rapamycin.

Table 5 Expression of immunohistochemical markers in categories of histological grade for primary hepatocellular carcinoma cases *n* (%)

IHC marker	Cut-off	Cases/valid samples			<i>P</i> value
		Grades 1 + 2	Grade 3	Grade 4	
EGFR overexpression	$\geq 200/300^1$	14 (67) ^a	12 (27)	3 (30)	0.008 ^a
Ki67	$\geq 0.1\%$	6 (30) ^a	31 (74)	8 (80)	0.002 ^a
K19	> 1%	0 (0)	7 (17)	5 (45) ^a	0.005 ^a
Vimentin	> 0/300	0 (0)	3 (7)	1 (9)	0.465
Caspase 3 loss	$\leq 10/300$	3 (15)	16 (38)	8 (73) ^a	0.006 ^a
Cyclin D1	> 0.1%	4 (20)	9 (21)	3 (30)	0.847
mTOR expression	$\geq 100/300^1$	7 (37)	17 (45)	1 (10)	0.134
MET expression	$\geq 100/300^1$	8 (42) ^a	6 (14)	0 (0)	0.012 ^a
ERK1 overexpression	$\geq 200/300^1$	0 (0)	8 (20)	2 (18)	0.081
ERK2 overexpression	$\geq 200/300^1$	1 (5)	6 (14)	3 (30)	0.148
p53	$\geq 10\%$	5 (28)	16 (42)	7 (64)	0.164
Beta-catenin, membrane	$\geq 100/300^1$	13 (65)	25 (64)	4 (40)	0.343
Beta-catenin, nucleus	2 or 3+	0 (0)	1 (3)	1 (10)	0.352

¹Score value. ^a*P* < 0.05 (outliers in pairwise test). EGFR: Epidermal growth factor receptor; ERK: Extracellular signaling-related kinase; HCC: Hepatocellular carcinoma; IHC: Immunohistochemistry; MET: MET receptor tyrosine kinase; mTOR: Mechanistic target of rapamycin.

Table 6 General correlation between expression scores for immunohistochemical markers and categories of histological grade for primary hepatocellular carcinoma

	Grade	Ki67	K19	p53	ERK1	BcatN	ERK2	Vim	CKD1	BcatM	mTOR	MET	EGFR	Group No.
Ki67	0.43 ^a													Group 1
K19	0.37 ^b	0.03												
p53	0.36 ^c	0.29 ^d	0.08											
ERK1	0.34 ^c	0.39 ^b	0.37 ^b	0.17										
BcatN	0.23	0.44 ^a	0.13	0.24	0.18									
ERK2	0.19	0.63 ^a	0.22	-0.10	0.36 ^c	0.24								Group 2
Vim	0.14	0.11	0.51 ^a	0.02	0.31 ^c	0.08	0.14							
CKD1	0.03	0.46 ^a	-0.32 ^c	-0.10	0.03	0.26 ^d	0.29 ^d	-0.19						
BcatM	-0.08	0.26 ^d	-0.01	-0.23	0.17	-0.07	0.42 ^a	0.01	0.25 ^d					
mTOR	-0.24 ^d	-0.02	-0.20	-0.21	0.25 ^d	-0.03	0.13	-0.13	0.17	0.44 ^a				
MET	-0.32 ^c	0.16	-0.20	-0.35 ^c	-0.01	0.22	0.39 ^b	-0.18	0.38 ^b	0.18	0.40 ^b			Group 3
EGFR	-0.34 ^c	0.00	-0.14	-0.13	-0.04	-0.24	0.22	-0.07	0.13	0.20	0.11	0.23		
Casp3	-0.42 ^a	-0.18	-0.25 ^d	-0.46 ^a	-0.19	-0.06	0.06	-0.27 ^d	0.19	0.16	0.32 ^b	0.41 ^a	0.35 ^c	

^a*P* < 0.0005, ^b*P* ≤ 0.001, ^c*P* < 0.01, ^d*P* < 0.05 by Spearman's rho. Cases with positivity (group 1) and negativity (group 3) for immunohistochemical markers as associated with the histological grade and cell proliferation; Cases in group 2 showed intermediate features, between groups 1 and 3. BcatM: Membrane beta-catenin; BcatN: Nuclear beta-catenin; Casp3: Caspase 3; CKD1: Cyclin D1; EGFR: Epidermal growth factor receptor; HCC: Hepatocellular carcinoma; mTOR: Mechanistic target of rapamycin; Vim: Vimentin.

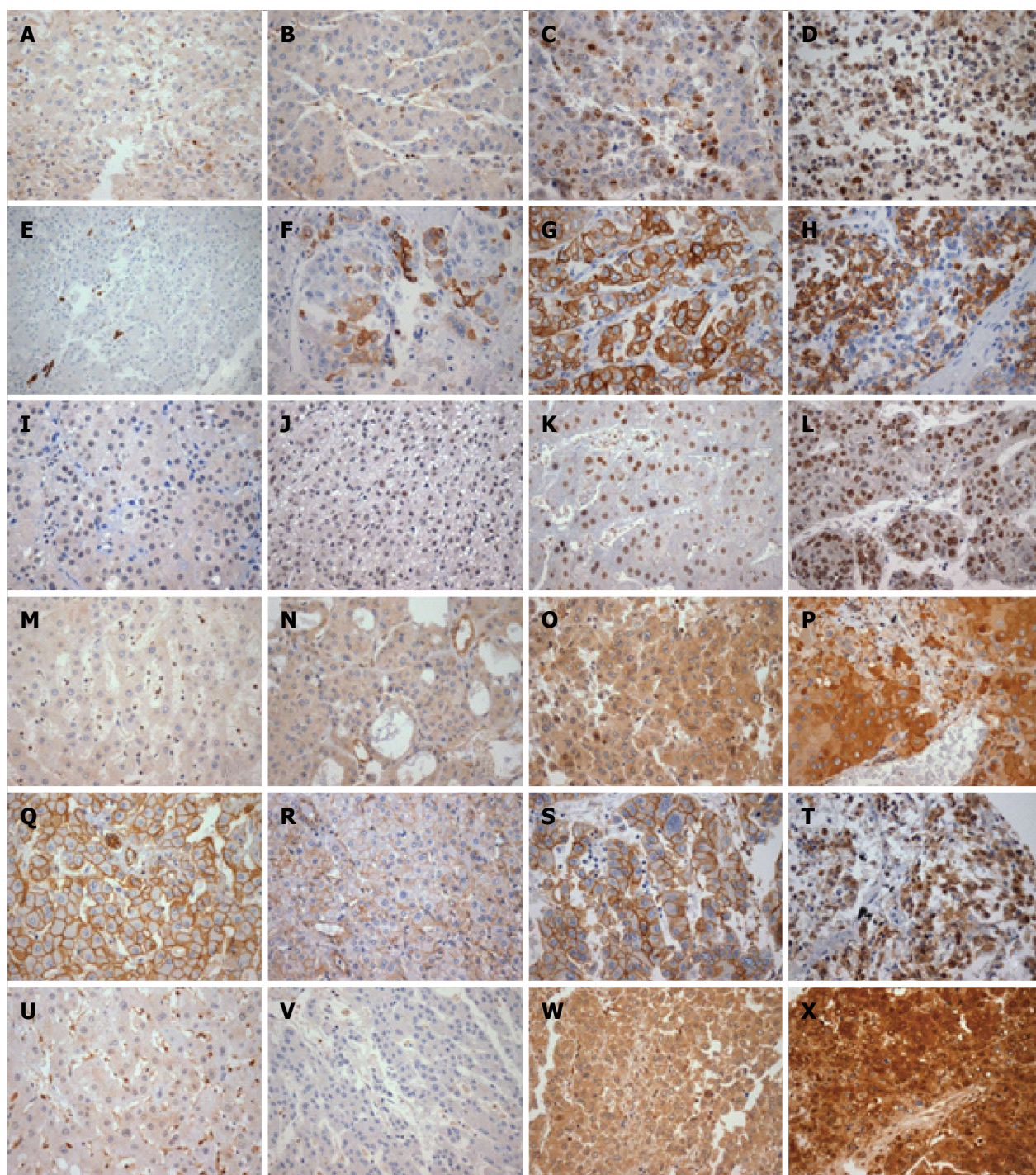


Figure 1 Panel of immunohistochemical markers. Ki67 in A: Cirrhosis (low); B: Grade 2 (low); C: Grade 3 (moderate); D: Grade 4 (high). K19 in E: Cirrhosis (negative in hepatocytes); F: Grade 3 (focal); G: Grade 3 (moderate); H: Grade 4 (moderate); p53 in I: Cirrhosis (negative); J: Grade 2 (focal); K: Grade 3 (moderate); L: Grade 3 (diffuse). ERK1 in M: Cirrhosis (negative); N: Grade 2 (weak); O: Grade 3 (moderate); P: Grade 3 (strong). Beta-catenin in Q: Cirrhosis (strong membrane expression); R: Grade 2 (weak membrane); S: Grade 3 (strong membrane); T: Grade 4 (nuclear expression). ERK2 in U: Cirrhosis (negative in hepatocytes and positive in endothelial cells); V: Grade 2 (negative); W: Grade 3 (moderate); X: Grade 3 (strong). Original magnification $\times 400$.

DISCUSSION

A few recent studies have sought to translate genomic data into panels of immunohistochemical markers, aiming to generate risk models for survival and prognostic evaluation of HCC^[16,17]. Although immunohistochemical studies do not always directly reflect the underlying molecular mechanisms of a

given tumor, this cross-sectional analysis in a large post-mortem series supports the feasibility of such a strategy and reinforces the paramount importance of the histological grade.

Overexpression of EGFR has been reported in HCC and the surrounding chronically inflamed tissue. In our autopsy cohort, EGFR overexpression was found to be related to more differentiated HCC. Previous

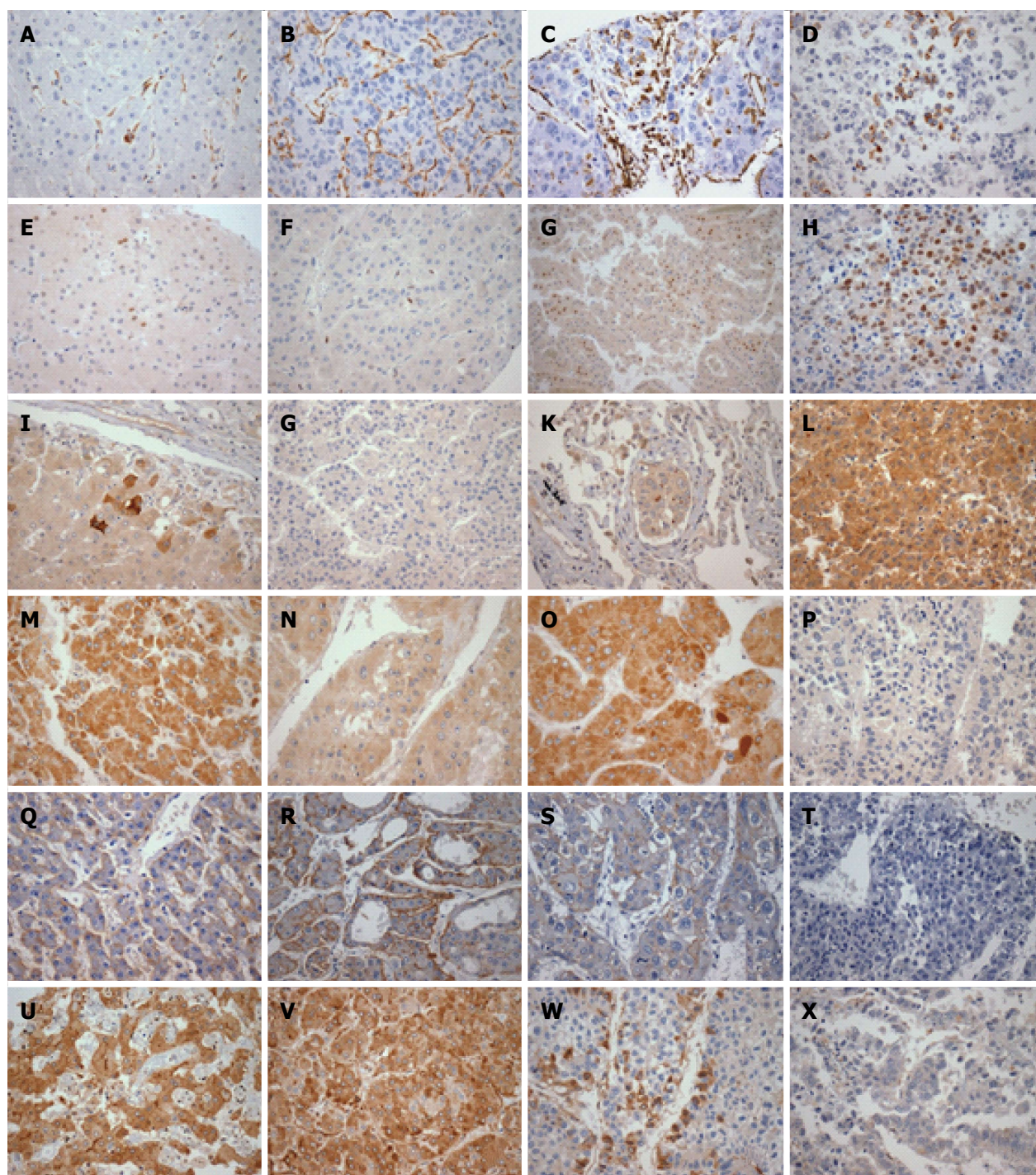


Figure 2 Panel of immunohistochemical markers. Vimentin in A: Cirrhosis (negative in hepatocytes and positive in sinusoids); B: Grade 2 (negative in hepatocytes and positive in sinusoids); C: Grade 3 (focally positive); D: Grade 4 (focally positive in small cell component). Cyclin-D1 in E: Cirrhosis (focal); F: Grade 2 (focal); G: Grade 3 (moderate); H: Grade 4 (moderate); mTOR in I: Cirrhosis (focal); J: Grade 2 (negative); K: Metastasis grade 3 (weak); L: Grade 4 (strong). MET in M: Cirrhosis (moderate); N: Grade 2 (weak); O: Grade 3 (moderate); P: Grade 3 (negative). EGFR in Q: Cirrhosis (moderate - score 140); R: Grade 2 (score 200); S: Grade 3 (score 60); T: Grade 4 (score 0). Caspase 3 in U: Cirrhosis (moderate); V: Grade 2 (moderate); W: Grade 3 (weak); X: Grade 3 (negative/loss). Original magnification $\times 400$.

studies have presented conflicting results on this topic, either reporting detection of higher EGFR expression in high-grade tumors^[18,19] or demonstrating a complete absence of any association^[20,21]. Our findings are similar to those described by Morimitsu *et al.*^[22], who showed a progressive loss of EGFR expression in less differentiated HCC in surgical specimens.

Our study also found that expression of Ki67, cyclin D1 and caspase 3 indicate higher proliferative activity and lower rates of apoptosis in more aggressive tumor populations, especially in metastases. However, these findings may not accurately reflect the histological grade since the metastases samples examined showed morphological similarities to the intrahepatic tumor

samples examined. Similarly, lower expression of caspase 3 has been previously reported as present in less differentiated prostate adenocarcinomas^[23]. Conversely, Persad *et al.*^[24] showed increased expression of caspase 3 in 52% of resected HCC samples, a distinguishing feature from the surrounding non-HCC tissue. The results from our study, presented herein, probably reflect a larger sample of poorly differentiated HCC with a higher proportion of caspase 3 loss.

The higher rates of K19 and vimentin expression we observed in metastatic HCC suggest a more aggressive behavior of K19-positive HCC; this finding could serve to reinforce previous evidence that K19 expression is a "progenitor cell feature", while vimentin expression could denote epithelial-mesenchymal transition^[25-27].

A subpopulation of HCC has been reported as presenting constitutively increased expression of cyclin D1. Activation of the cyclin D1 promoter has also been characterized as one of the prime targets of the beta-catenin pathway. Joo *et al.*^[26] linked the overexpression of cyclin D1 to well-differentiated HCC that shows a low proliferation index as evidenced by detection of Ki67. The authors, however, did not identify an association between the expression of cyclin D1 and p53, as we observed in the current study. Instead, our results are similar to those described by Schmitt-Graeff *et al.*^[28], in which an association was found to exist between the expression of cyclin D1 and cell proliferation, but not with histological grade. Unlike that finding and those reported by Prange *et al.*^[29], we identified a weak correlation between the expression of cyclin D1 and the nuclear or membrane expression of beta-catenin. Our finding is in accordance with another study that identified an association between aberrant nuclear expression of beta-catenin and the Ki67 index^[30]. Dysregulation of the Wnt pathway may be more relevant in a type of HCC with a higher rate of cell proliferation, but which is distinct from tumors with stemness features.

Our study also showed that expression of ERK1 associated with histological grade, as well as with the expression of K19 and vimentin. On the other hand, expression of ERK2 correlated to that of cyclin D1, membrane beta-catenin and MET, but not to histological grade. These findings suggest that ERK1 is preferentially expressed in HCC that has a stem-cell phenotype, while ERK2 is preferentially expressed in HCC that has a dysregulated Wnt pathway and/or dysregulated MET pathway. It has been reported that activation of kinases in HCC indicates aggressive behavior and may represent an independent prognostic factor for progression to HCC in HCV-infected individuals^[31]. This activation seems to be related to the mammalian sterile-20-like kinase 4, which functions as an enhancer of cell proliferation and invasion through its ability to promote the process of epithelial-mesenchymal transition^[32]. ERK2 may play a more prominent role in the proliferation of hepatocytes,

while ERK1 may play a pro-apoptotic role^[33]. An HCC type having a progenitor cell phenotype may present mechanisms of escaping the pro-apoptotic action of ERK1, which are presumably related to the mutation of p53. The overexpression of ERK1/ERK2 kinases in this study's series of advanced cases was not related to EGFR overexpression.

Autopsy studies may have limitations that should be considered when interpreting results, particularly those related to autolytic changes that may affect immunoreactivity. In our institution, corpses are refrigerated and medical autopsies are performed as soon as all technical and legal procedures are addressed and resolved. Nevertheless, we sought to minimize the effects of autolytic changes by excluding samples with morphological features of poor preservation, specifically by examining vimentin staining (comparing to positive and negative controls) to assure that protein preservation was good enough to yield reliable immunohistochemical studies^[34].

In conclusion, in the present autopsy cohort we found relevant associations between immunohistochemical markers with morphological features of tumors, especially that of histological grading of HCC^[35]. The expression of K19, p53, ERK1, ERK2, vimentin and nuclear beta-catenin showed an association with pathological markers of higher-grade tumors, as opposed to cases expressing/over-expressing EGFR, MET and caspase 3. Further studies in the clinical setting are warranted to assess the prognostic value of this approach not only in advanced cases but also in early stages of HCC, especially in surgical resections or in explants.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is an increasingly prevalent cancer and many patients are still diagnosed in the advanced stages, and usually as a complication of other chronic liver disease. In Brazil, chronic viral hepatitis and alcohol intake are the major related causes of HCC. Autopsy studies of advanced tumors provide a unique opportunity to assess important pathological aspects of progression and dissemination patterns of HCC.

Research frontiers

Recent studies have sought to translate genomic data into panels of immunohistochemical markers, aiming to generate risk models for survival and prognostic evaluation of HCC. Morphological and immunohistochemical data may be incorporated in clinicopathological indices to predict HCC molecular classification and prognosis. The primary objective of the current study was to assess the distribution of proteins coded by genes reported as relevant to molecular classification of HCC based upon a detailed clinicopathological analysis of an autopsy cohort.

Innovations and breakthroughs

This retrospective cross-sectional analysis of a large post-mortem cohort series shows that a panel of immunohistochemical markers may be related to different pathways underlying progression and metastasis of HCC, and that the Edmondson-Steiner's tumor grade may reflect currently recognized molecular subclasses of the disease. It also provides a detailed description of clinicopathological data in a unique autopsy series of HCC patients in Brazil.

Applications

The findings from this study form a foundation for further investigations of morpho-molecular correlations, not only in advanced cases of HCC but also in early stage cases and especially in surgical resections or explants.

Terminology

Edmondson-Steiner's tumor grade classifies HCC on a scale from 1 to 4, based on nuclear and cellular atypia; described in 1954, this system is the most widely accepted for tumor grading of HCC. Immunohistochemistry technique is used to detect cell or tissue antigens in two phases: (1) slide preparation, which involves specimen fixation, tissue processing and the immunohistochemical reaction (antigen retrieval, blocking of non-specific interaction sites, blocking of endogenous peroxidase, incubation with primary antibody, detection of immunoreactivity and counterstaining, as well as slide mounting); and (2) interpretation and quantification of the detected expression. Tissue microarrays contain many small representative cylindrical cores of tissue from different cases assembled on a single paraffin block and correspondent histological slide, thereby facilitating high-throughput analysis of multiple specimens simultaneously; it is produced by extracting the tissue cores from different (hundreds of) paraffin-donor blocks and re-embedding these into a single recipient block at defined array coordinates, thereby permitting simultaneous analysis of protein expression under standardized conditions on a single glass slide and also providing maximal preservation and use of limited and irreplaceable archival tissue samples.

Peer-review

The authors have conducted a detailed and elegantly illustrated histological study showing that groups of markers may be related to different pathways underlying HCC progression and metastasis, and that Edmondson-Steiner's tumor grade may reflect currently recognized molecular subclasses of HCC.

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Case Control Study

Effect of dietary vitamin C on gastric cancer risk in the Korean population

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Abstract

AIM: To investigate the effects of dietary vitamin C and foods containing vitamin C on gastric cancer risk.

METHODS: Our study included 830 control subjects and 415 patients. Data regarding demographics, medical history, and lifestyle, including dietary and nutrient intake, were collected using reliable self-administered questionnaires. Dietary intake information was collected from the participants using a food frequency questionnaire that has been previously reported as reliable and valid. A rapid urease test and a histological evaluation were used to determine the presence of *Helicobacter pylori* (*H. pylori*) infection. Twenty-three vitamin C-contributing foods were selected, representing over 80% of the cumulative vitamin C contribution.

RESULTS: In analyses adjusted for first-degree family history of gastric cancer, education level, job, household income, smoking status, and regular exercise, an inverse

association between vitamin C intake and gastric cancer risk was observed for the highest (≥ 120.67 mg/d) vs the lowest (< 80.14 mg/d) intake category [OR (95%CI): 0.64 (0.46-0.88)], with a significant trend across the three intake categories ($P = 0.007$). No protective effect of vitamin C was detected after stratification by gender. No effect of vitamin C intake on the gastric cancer incidence was found in either men or women infected with *H. pylori*. Vitamin C-contributing foods, including cabbage [0.45 (0.32-0.63), 0.50 (0.34-0.75), 0.45 (0.25-0.81)], strawberries [0.56 (0.40-0.78), 0.49 (0.32-0.74), 0.52 (0.29-0.93)], and bananas [0.40 (0.29-0.57), 0.41 (0.27-0.62), 0.34 (0.19-0.63)], were protective factors against the risk of gastric cancer based on the results of the overall adjusted analyses and the results for men and women, respectively.

CONCLUSION: A protective effect of vitamin C and vitamin C-contributing foods against gastric cancer was observed. Further studies using larger sample sizes are required to replicate our results.

Key words: Vitamin C; Vitamin C-contributing foods; *Helicobacter pylori*; Gastric cancer; Korean population

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Core tip: An increased intake of vitamin C and vitamin C-contributing foods, including vegetables and fruits, may protect individuals against the risk of gastric cancer. However, we have no sufficient evidence to support the hypothesis that vitamin C has protective effect against gastric cancer in individuals infected with *Helicobacter pylori*.

Hoang BV, Lee J, Choi IJ, Kim YW, Ryu KW, Kim J. Effect of dietary vitamin C on gastric cancer risk in the Korean population. *World J Gastroenterol* 2016; 22(27): 6257-6267 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6257.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6257>

INTRODUCTION

Although the incidence and mortality rates of gastric cancer have decreased worldwide, stomach cancer remains the fifth most common cancer and the third leading cause of cancer death in both sexes worldwide^[1]. Being the most common cancer among men in Korea, gastric cancer had 41.3 and 7.8 per 100000 persons in the estimated age-standardized incidence and mortality rates in 2015, respectively^[2].

Dietary habits and nutrient intake play important roles in the prevention and etiology of gastric cancer^[3]. According to the World Cancer Research Fund and the American Institute for Cancer Research report, increased consumption of non-starchy vegetables and fruits may decrease the risk of gastric cancer, whereas

salt and salted foods may be the risk factors of gastric cancer. Additionally, a number of other foods may associate with gastric cancer. However, no specific constituent of these foods has yet been identified to explain these reported associations^[3]. Being one of the most common antioxidants found in fruits and vegetables, vitamin C may have a chemopreventive effect^[4]. Vitamin C protects cells from oxidative DNA damage, thereby blocking carcinogenesis^[5]. Additionally, the protective effect of vitamin C is supported by many observational studies and meta-analyses^[6-15]. However, some observational studies did not successfully demonstrate a significant association between vitamin C intake and gastric cancer^[16-18]. To date, the association between vitamin C intake and gastric cancer risk has been inconsistent.

Helicobacter pylori (*H. pylori*) is classified as a cause of stomach cancer in a monograph from the International Agency for Research on Cancer (IARC)^[19,20]. Epidemiological studies in humans have linked vitamin C deficiency to more severe *H. pylori*-associated gastritis and a higher risk of gastric cancer^[21,22]. Furthermore, reduced vitamin C levels in the gastric juice and plasma in *H. pylori*-infected patients returned to normal levels after *H. pylori* eradication^[7,22-24]. Therefore, *H. pylori*-induced gastric cancer may be prevented by an appropriate diet.

We performed a case-control study to investigate the effects of dietary vitamin C and vitamin C-contributing foods on gastric cancer risk.

MATERIALS AND METHODS

Study population

This study is an expansion of two previously published case-control studies^[25,26]. The control and case groups were obtained from the National Cancer Center Hospital in South Korea between March 2011 and December 2014. Individuals who were histologically confirmed as early gastric cancer patients within the preceding three months at the Center for Gastric Cancer were included in the case group. Early gastric carcinoma is an invasive carcinoma confined to the mucosa and/or submucosa, with or without lymph node metastases, irrespective of the tumor size^[27]. Patients in the case group did not have diabetes mellitus, a history of cancer within the past five years, advanced gastric cancer, or severe systemic or mental disease, nor were they women who were pregnant or breastfeeding. We selected the control group from patients undergoing health-screening examinations at the Center for Cancer Prevention and Detection at the same hospital.

In total, 1727 participants were recruited, with 1227 in the control group and 500 in the case group; 1671 individuals provided data through a food frequency questionnaire (FFQ) and a self-administered questionnaire. Participants with a total energy intake of < 500 kcal or ≥ 4000 kcal ($n = 15$) were excluded

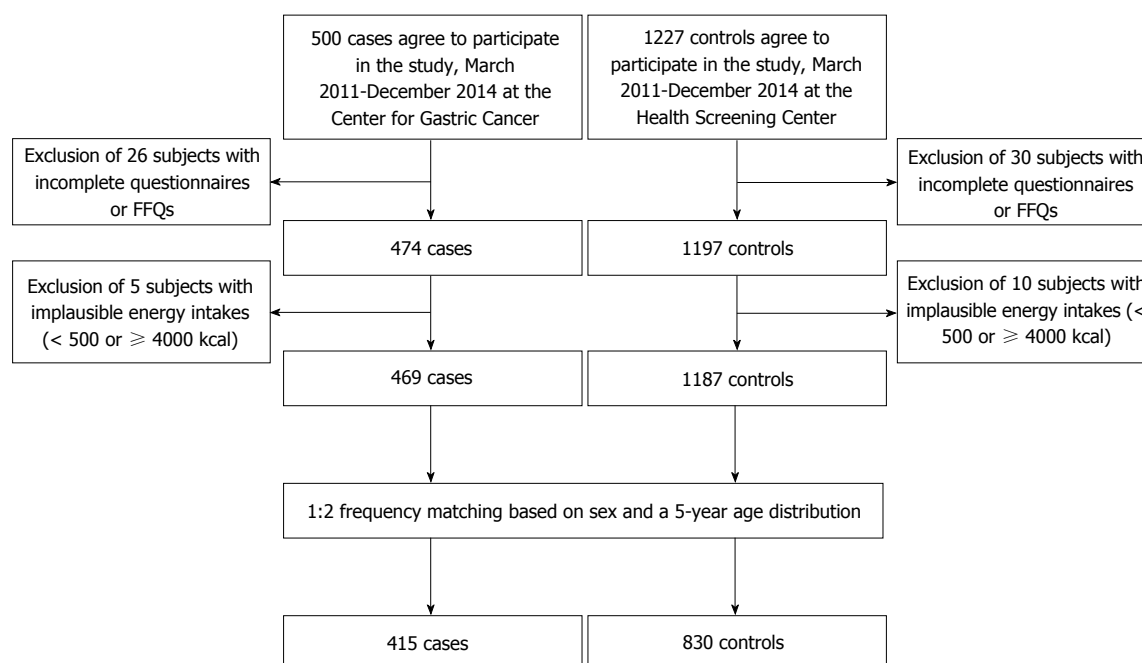


Figure 1 Flow diagram for included participants.

because of the implausibility of the data. Of the 1656 individuals remaining, the control and case subjects were frequency-matched by age (within 5 years) and sex at a ratio of 2:1 (controls:cases). The final analysis consisted of 1245 participants, including 830 controls and 415 cases (men, 810; women, 435; Figure 1). Our study was approved by the Institutional Review Board of the National Cancer Center [IRB Number: NCCNCS-11-438]. We collected written informed consent from all participants.

Data collection

The participants were asked to complete a self-administered questionnaire that included demographic, lifestyle, and medical history information. Dietary intake information was collected from the participants using the FFQ, which has been previously reported as a reliable and valid questionnaire^[28]. The FFQ contains nine food consumption frequency categories (never or rarely, once a month, 2 or 3 times a month, once or twice a week, 3 or 4 times a week, 5 or 6 times a week, once a day, twice a day, and 3 times a day) and three portion size categories (small, medium, and large) for specific food items consumed within the past 12 mo. We used CAN-Pro 4.0 (Computer Aided Nutritional Analysis Program, The Korean Nutrition Society, Seoul, Korea) to calculate the average daily nutrient intake for each participant, and we summed the amounts of vitamin C obtained from various food groups to compute the vitamin C intake (mg/d). A rapid urease test and histological evaluation were used to assess *H. pylori* infection.

Statistical analysis

We used *t* tests and χ^2 tests for continuous and

categorical variables, respectively, to compare the characteristics of the control and case groups. We conducted a contribution analysis to select vitamin C-contributing foods, which were ranked by the percentage of the total vitamin C intake that they provide for the population as a whole. A total of 23 vitamin C-contributing foods were selected, representing over 80% of the cumulative contribution. To compare the difference in dietary vitamin C intake and vitamin C-contributing foods, consumption was adjusted for total energy intake using the linear residual regression method^[29]. The intake levels of vitamin C and vitamin C-contributing foods were categorized into tertiles according to the distribution of the control group. The lowest tertile group was used as the reference group. The median values of each tertile category of the dietary vitamin C intake and vitamin C-contributing foods were used as a continuous variable to test for trends.

The association between dietary factors and gastric cancer risk was assessed using an analysis with logistic regression models adjusted for potential confounding variables, and the odds ratios (OR) and their 95% confidence intervals (CIs) were calculated. Multivariate models were adjusted for first-degree family history of gastric cancers (yes, no), education level (middle school or less, high school, and college or more), job (managers and professionals, clerical, sales and service, production workers and laborers, and not in the labor force), monthly household income (< 2000000 KRW, 2000000-4000000 KRW, \geq 4000000 KRW), smoking status (nonsmoker, ex-smoker, and current smoker), regular exercise (yes, no), and *H. pylori* infection (positive, negative). SAS 9.3 software (SAS Institute., Cary, NC, United States) was used to perform the calculations, and a two-sided *P* value less

Table 1 General characteristics of the study subjects

	Total (<i>n</i> = 1245)			Men (<i>n</i> = 810)			Women (<i>n</i> = 435)		
	Case (<i>n</i> = 415)	Control (<i>n</i> = 830)	<i>P</i> value ¹	Case (<i>n</i> = 270)	Control (<i>n</i> = 540)	<i>P</i> value	Case (<i>n</i> = 145)	Control (<i>n</i> = 290)	<i>P</i> value
Age (yr), mean ± SD	53.8 ± 9.3	53.7 ± 9.0	0.892	54.9 ± 8.7	54.8 ± 8.4	0.905	51.7 ± 10.0	51.6 ± 9.8	0.942
Body mass index (kg/m ²)									
< 23	159 (38.3)	314 (37.8)	0.975	91 (33.7)	161 (29.8)	0.509	68 (46.9)	153 (52.8)	0.533
23-25	122 (29.4)	249 (30.0)		78 (28.9)	170 (31.5)		44 (30.3)	79 (27.2)	
≥ 25	133 (32.1)	266 (32.1)		101 (37.4)	209 (38.7)		32 (22.1)	57 (19.7)	
First-degree family history of gastric cancer									
No	332 (80.0)	725 (87.4)	0.001	209 (77.4)	464 (85.9)	0.002	123 (84.8)	261 (90.0)	0.114
Yes	82 (19.8)	103 (12.4)		60 (22.2)	74 (13.7)		22 (15.2)	29 (10.0)	
Marital status									
Married	361 (87.0)	716 (86.3)	0.611	243 (90.0)	478 (88.5)	0.475	118 (81.4)	238 (82.1)	0.975
Other	52 (12.5)	113 (13.6)		26 (9.6)	61 (11.3)		26 (17.9)	52 (17.9)	
Education level									
Less than middle school	142 (34.2)	119 (14.3)	< 0.001	91 (33.7)	71 (13.2)	< 0.001	51 (35.2)	48 (16.6)	< 0.001
High school	174 (41.9)	253 (30.5)		112 (41.5)	140 (25.9)		62 (42.8)	113 (39.0)	
College or higher	97 (23.4)	426 (51.3)		66 (24.4)	301 (55.7)		31 (21.4)	125 (43.1)	
Job									
Managers and professionals	70 (16.9)	156 (18.8)	0.001	59 (21.9)	117 (21.7)	0.010	11 (7.6)	39 (13.5)	0.002
Clerical, sales and service workers	122 (29.4)	266 (32.1)		81 (30.0)	203 (37.6)		41 (28.3)	63 (21.7)	
Production workers, and laborers	104 (25.1)	128 (15.4)		83 (30.7)	111 (20.6)		21 (14.5)	17 (5.9)	
Not in the labor force	117 (28.2)	277 (33.4)		46 (17.0)	106 (19.6)		71 (49.0)	171 (59.0)	
Monthly household income ²									
< 200	133 (32.1)	149 (18.0)	< 0.001	85 (31.5)	85 (15.7)	< 0.001	48 (33.1)	64 (22.1)	0.016
200-400	148 (35.7)	341 (41.1)		106 (39.3)	232 (43.0)		42 (29.0)	109 (37.6)	
≥ 400	96 (23.1)	273 (32.9)		55 (20.4)	168 (31.1)		41 (28.3)	105 (36.2)	
Alcohol consumption									
Non-drinker	119 (28.7)	236 (28.4)	0.243	44 (16.3)	89 (16.5)	0.282	75 (51.7)	147 (50.7)	0.819
Ex-drinker	41 (9.9)	60 (7.2)		33 (12.2)	47 (8.7)		8 (5.5)	13 (4.5)	
Current drinker	254 (61.2)	534 (64.3)		193 (71.5)	404 (74.8)		61 (42.1)	130 (44.8)	
Smoking status									
Non-smoker	167 (40.2)	384 (46.3)	< 0.001	39 (14.4)	106 (19.6)	< 0.001	128 (88.3)	278 (95.9)	0.021
Ex-smoker	119 (28.7)	284 (34.2)		110 (40.7)	277 (51.3)		9 (6.2)	7 (2.4)	
Current-smoker	128 (30.8)	162 (19.5)		121 (44.8)	157 (29.1)		7 (4.8)	5 (1.7)	
Regular exercise									
No	268 (64.6)	361 (43.5)	< 0.001	161 (59.6)	234 (43.3)	< 0.001	107 (73.8)	127 (43.8)	< 0.001
Yes	147 (35.4)	466 (56.1)		109 (40.4)	303 (56.1)		38 (26.2)	163 (56.2)	
<i>H. pylori</i> infection									
Negative	33 (8.0)	320 (38.6)	< 0.001	18 (6.7)	187 (34.6)	< 0.001	15 (10.3)	133 (45.9)	< 0.001
Positive	382 (92.1)	486 (58.6)		252 (93.3)	333 (61.7)		130 (89.7)	153 (52.8)	

¹*P* values were calculated using the *t* test (for continuous variables) or χ^2 test (for categorical variables); ²Unit is 10000 won in Korean currency. Values are expressed as the mean ± SD (range) or *n* (%).

than 0.05 was considered statistically significant.

RESULTS

General characteristics

Table 1 shows the distribution of 830 control subjects and 415 patients with gastric cancer according to general characteristics. Gastric cancer patients who had a higher family history of gastric cancer (*P* = 0.001) and tended to have a lower education level (*P* < 0.001), lower levels of employment (*P* < 0.001) and household income (*P* < 0.001) reported using more tobacco (*P* < 0.001), performing less regular exercise (*P* < 0.001), and having a higher proportion of *H. pylori* infection (*P*

< 0.001). Compared with the control group, both men and women in the case group had a lower education level, job, and household income, used more tobacco, performed less regular exercise, and had a higher proportion of *H. pylori* infection. In particular, the men in the case group had a higher percentage of family history of gastric cancer than the men in the control group (*P* = 0.002).

Vitamin C and vitamin C-contributing food consumption is described in Table 2. Lower vitamin C intake (*P* < 0.001), increased consumption of potatoes and starches (*P* = 0.013) and fruits (*P* < 0.001), and higher energy intake (*P* < 0.001) were found in the case group. In general, the case group consumed less

Table 2 Comparison of intakes of vitamin C and vitamin C contributing foods¹

Food (g/d) (mean ± SD)	Total (n = 1245)			Men (n = 810)			Women (n = 435)		
	Case (n = 415)	Control (n = 830)	P value ²	Case (n = 270)	Control (n = 540)	P value	Case (n = 145)	Control (n = 290)	P value
Energy (Kcal/d)	1924.1 ± 612.9	1713.6 ± 545.5	< 0.001	2038.5 ± 634.8	1760.6 ± 541.5	< 0.001	1711.1 ± 507.0	1626.0 ± 543.1	0.116
Vitamin C (mg/d)	96.1 ± 50.5	108.4 ± 56.1	< 0.001	89.0 ± 45.3	97.1 ± 44.2	0.014	109.3 ± 56.7	129.5 ± 68.5	0.001
Potatoes and starches	39.3 ± 38.1	45.5 ± 45.4	0.013	32.8 ± 34.6	40.3 ± 36.5	0.005	51.5 ± 41.2	55.1 ± 57.4	0.456
Potatoes	32.3 ± 34.0	35.0 ± 35.0	0.209	27.7 ± 32.2	32.7 ± 30.3	0.035	41.0 ± 35.7	39.1 ± 42.1	0.635
Sweet potatoes	24.8 ± 210.0	42.3 ± 234.4	0.183	8.2 ± 41.9	15.1 ± 47.1	0.030	55.6 ± 349.4	92.8 ± 386.8	0.329
Vegetables	327.4 ± 185.0	328.2 ± 166.2	0.947	318.4 ± 177.5	320.5 ± 157.4	0.873	344.3 ± 197.7	342.5 ± 180.8	0.926
Korean cabbage kimchi	99.3 ± 71.0	96.1 ± 69.3	0.450	94.9 ± 66.6	97.3 ± 69.0	0.639	107.4 ± 78.1	93.8 ± 70.0	0.068
Green pepper	7.3 ± 11.0	7.9 ± 10.6	0.347	6.4 ± 6.8	7.5 ± 9.5	0.044	9.0 ± 16.0	8.6 ± 12.5	0.769
Radish	20.3 ± 17.5	21.2 ± 18.3	0.375	20.3 ± 16.8	21.5 ± 17.5	0.348	20.3 ± 18.8	20.8 ± 19.6	0.801
Spinach	9.9 ± 21.6	10.7 ± 22.8	0.572	8.0 ± 18.5	9.1 ± 17.3	0.380	13.6 ± 26.2	13.6 ± 30.4	0.994
Radish kimchi	27.1 ± 80.2	28.5 ± 58.9	0.765	20.7 ± 40.1	26.8 ± 52.3	0.065	39.1 ± 123.6	31.5 ± 69.6	0.491
Cabbage	6.6 ± 13.9	13.4 ± 27.1	< 0.001	4.6 ± 10.3	9.7 ± 20.0	< 0.001	10.3 ± 18.3	20.4 ± 35.8	< 0.001
Chonggak kimchi	15.7 ± 44.9	16.5 ± 33.6	0.769	12.1 ± 23.1	15.6 ± 29.9	0.072	22.4 ± 68.7	18.1 ± 39.4	0.486
Zucchini	18.0 ± 22.1	17.5 ± 20.4	0.711	16.1 ± 20.3	15.4 ± 18.3	0.650	21.6 ± 24.9	21.5 ± 23.2	0.956
Chinese cabbage	22.3 ± 118.0	32.7 ± 142.8	0.172	20.7 ± 132.2	34.0 ± 164.8	0.217	25.1 ± 85.7	30.3 ± 88.5	0.564
Lettuce	7.2 ± 10.1	9.3 ± 15.5	0.004	6.1 ± 8.8	7.6 ± 10.7	0.039	9.1 ± 11.9	12.4 ± 21.4	0.040
Onion	14.5 ± 8.7	15.0 ± 9.2	0.375	13.4 ± 7.6	14.3 ± 8.6	0.166	16.4 ± 10.2	16.3 ± 10.2	0.887
Mustard leaf kimchi	10.5 ± 61.5	14.2 ± 118.4	0.469	8.1 ± 62.4	8.2 ± 58.9	0.990	14.9 ± 59.8	25.4 ± 183.1	0.377
Green onion	4.8 ± 3.0	5.0 ± 3.3	0.410	4.9 ± 3.01	5.1 ± 3.3	0.410	4.7 ± 2.9	4.8 ± 3.5	0.811
Fruits	136.0 ± 165.8	191.8 ± 209.1	< 0.001	115.5 ± 149.4	152.0 ± 163.5	0.002	174.1 ± 187.4	266.0 ± 259.0	< 0.001
Mandarins	16.0 ± 26.3	23.2 ± 44.9	< 0.001	12.9 ± 22.6	14.6 ± 23.4	0.319	21.8 ± 31.5	39.2 ± 66.0	< 0.001
Strawberries	5.2 ± 8.7	8.8 ± 15.8	< 0.001	4.3 ± 7.7	6.7 ± 11.2	< 0.001	7.0 ± 10.0	12.6 ± 21.4	< 0.001
Orange juice	8.9 ± 22.2	20.8 ± 56.1	< 0.001	6.4 ± 14.2	16.9 ± 54.8	< 0.001	13.6 ± 31.6	28.0 ± 57.8	0.001
Watermelon	13.4 ± 21.7	21.0 ± 69.4	0.004	11.3 ± 21.2	15.3 ± 36.5	0.050	17.5 ± 22.2	31.8 ± 105.6	0.028
Apples	30.4 ± 57.1	52.3 ± 89.7	< 0.001	23.8 ± 49.1	42.7 ± 77.3	< 0.001	42.7 ± 68.1	70.2 ± 107.0	0.001
Persimmons	17.4 ± 110.5	20.3 ± 57.6	0.617	17.2 ± 133.6	12.9 ± 43.3	0.603	17.8 ± 41.9	34.1 ± 75.5	0.004
Bananas	10.5 ± 26.5	20.3 ± 40.9	< 0.001	7.3 ± 16.0	15.2 ± 28.9	< 0.001	16.4 ± 38.5	29.8 ± 55.8	0.004
Citrus tea	23.6 ± 142.9	58.5 ± 729.7	0.184	11.8 ± 62.8	18.1 ± 67.4	0.201	45.5 ± 225.0	133.7 ± 1228.9	0.237

¹Adjusted for total energy intake using the residuals method; ²P values were calculated with the *t* test.

cabbage ($P < 0.001$), lettuce ($P = 0.004$), mandarins ($P < 0.001$), strawberries ($P < 0.001$), orange juice ($P < 0.001$), watermelon ($P = 0.004$), apples ($P < 0.001$), and bananas ($P < 0.001$) than the control group. Compared with the control group, the men and women in the case group also consumed less vitamin C, cabbage, lettuce, fruits, strawberries, orange juice, watermelon, apples, and bananas. Some gender differences in vitamin C-contributing food consumption were found in both the case and control groups. The men in the case group consumed more energy ($P < 0.001$) and fewer starches ($P = 0.005$), potatoes ($P = 0.035$), sweet potatoes ($P = 0.030$), and green peppers ($P = 0.044$) than the control group. The women in the case group consumed fewer mandarins ($P < 0.001$) and persimmons ($P = 0.004$) than the women in the control group.

Vitamin C intake and the risk of gastric cancer

Table 3 reports the ORs and corresponding 95% CIs for vitamin C intake. Vitamin C intake exhibited was negatively associated with gastric cancer in both the unadjusted model [OR (95%CI): 0.53 (0.40-0.71), P for trend < 0.001] and the adjusted model (family history of gastric cancer, education level, job, household income, smoking status, and regular exercise; 0.64 (0.46-0.88), P for trend = 0.007. However, the association was marginally significant

after an additional adjustment for *H. pylori* status [0.71 (0.50-1.00), P for trend = 0.052]. No protective effect of vitamin C was observed in either gender as a result of the adjusted model.

The results were stratified by *H. pylori* status and sex in the present study. In the crude model, vitamin C intake was a protective factor against gastric cancer for participants infected with *H. pylori* [0.62 (0.45-0.87), P for trend = 0.006]. However, the association was weakened after an adjustment for confounding factors [0.74 (0.51-1.08), P for trend = 0.116]. No effect of vitamin C intake on the gastric cancer incidence was observed for both either men or women infected with *H. pylori* (data not shown).

Vitamin C - contributing food consumption and the risk of gastric cancer

Table 4 shows the association between vitamin C-contributing food consumption and the gastric cancer risk. Overall, the consumption of total fruit [0.57 (0.41-0.81)], sweet potatoes [0.62 (0.44-0.87)], cabbage [0.45 (0.32-0.63)], Chinese cabbage [0.58 (0.41-0.81)], lettuce [0.67 (0.49-0.93)], strawberries [0.56 (0.40-0.78)], orange juice [0.61 (0.44-0.85)], watermelon [0.69 (0.50-0.95)], apples [0.60 (0.43-0.85)], persimmons [0.56 (0.40-0.78)], and bananas [0.40 (0.29-0.57)] protects against gastric cancer based on the results of the adjusted model.

Table 3 ORs and 95%CI of gastric cancer by tertiles of dietary vitamin C

	Range (mg/d)	No. of controls/cases	Model I OR (95%CI)	Model II OR (95%CI)	Model III OR (95%CI)
Total (<i>n</i> = 1245)					
T1	< 80.14	276/186	1	1	1
T2	80.14-120.67	277/130	0.70 (0.53-0.92)	0.81 (0.59-1.10)	0.81 (0.58-1.12)
T3	≥ 120.67	277/99	0.53 (0.40-0.71)	0.64 (0.46-0.88)	0.71 (0.50-1.00)
<i>P</i> for trend ¹			< 0.001	0.007	0.052
Men (<i>n</i> = 810)					
T1	< 73.18	180/107	1	1	1
T2	73.18-110.59	180/93	0.87 (0.62-1.23)	1.11 (0.75-1.64)	1.07 (0.70-1.61)
T3	≥ 110.59	180/70	0.65 (0.45-0.94)	0.78 (0.52-1.18)	0.91 (0.59-1.41)
<i>P</i> for trend			0.022	0.229	0.659
Women (<i>n</i> = 435)					
T1	< 91.70	96/69	1	1	1
T2	91.70-139.52	97/45	0.65 (0.40-1.03)	0.81 (0.48-1.36)	0.85 (0.49-1.48)
T3	≥ 139.52	97/31	0.45 (0.27-0.74)	0.57 (0.32-1.00)	0.61 (0.34-1.12)
<i>P</i> for trend			0.002	0.051	0.109

¹Trends were calculated using the median intake for each dietary vitamin C category as a continuous variable: Model I : Unadjusted; Model II : Adjusted by first-degree family history of gastric cancer, education level, job, household income, smoking status, regular exercise; Model III: Additionally adjusted for *H. pylori* infection.

Table 4 ORs and 95%CI of gastric cancer by the highest tertile of vitamin C contributing food consumption

	Total (<i>n</i> = 1245)	<i>P</i> for trend ²	Men (<i>n</i> = 810)	<i>P</i> for trend	Women (<i>n</i> = 435)	<i>P</i> for trend
Potatoes and starches						
Model I OR (95%CI)	0.74 (0.54-1.59)	0.020	0.55 (0.37-0.82)	0.001	0.97 (0.60-1.57)	0.996
Model II OR (95%CI)	0.72 (0.52-1.01)	0.028	0.55 (0.36-0.85)	0.003	0.94 (0.55-1.60)	0.889
Model III OR (95%CI)	0.85 (0.59-1.21)	0.277	0.65 (0.41-1.03)	0.042	1.01 (0.57-1.79)	0.891
Total vegetable consumption						
Model I OR (95%CI)	0.87 (0.66-1.16)	0.366	0.91 (0.64-1.31)	0.593	0.86 (0.54-1.37)	0.549
Model II OR (95%CI)	0.91 (0.66-1.25)	0.575	1.01 (0.67-1.52)	0.955	0.83 (0.49-1.39)	0.496
Model III OR (95%CI)	0.96 (0.68-1.34)	0.800	1.09 (0.71-1.68)	0.744	0.82 (0.47-1.43)	0.494
Total fruit consumption						
Model I OR (95%CI)	0.41 (0.30-0.56)	< 0.001	0.52 (0.36-0.75)	0.001	0.34 (0.21-0.57)	< 0.001
Model II OR (95%CI)	0.57 (0.41-0.81)	0.002	0.73 (0.49-1.10)	0.148	0.52 (0.30-0.92)	0.032
Model III OR (95%CI)	0.59 (0.41-0.85)	0.005	0.73 (0.47-1.13)	0.179	0.57 (0.31-1.05)	0.089
Potatoes						
Model I OR (95%CI)	0.82 (0.61-1.10)	0.114	0.60 (0.41-0.87)	0.003	1.19 (0.73-1.93)	0.444
Model II OR (95%CI)	0.79 (0.57-1.09)	0.105	0.55 (0.36-0.85)	0.003	0.99 (0.57-1.70)	0.867
Model III OR (95%CI)	0.91 (0.64-1.29)	0.458	0.65 (0.41-1.02)	0.034	1.10 (0.61-1.97)	0.572
Sweet potatoes						
Model I OR (95%CI)	0.57 (0.42-0.77)	< 0.001	0.54 (0.37-0.80)	< 0.001	0.71 (0.44-1.16)	0.244
Model II OR (95%CI)	0.62 (0.44-0.87)	0.002	0.60 (0.39-0.92)	0.003	0.68 (0.39-1.18)	0.196
Model III OR (95%CI)	0.69 (0.48-1.00)	0.018	0.66 (0.42-1.05)	0.016	0.76 (0.42-1.37)	0.294
Korean cabbage kimchi						
Model I OR (95%CI)	1.08 (0.81-1.43)	0.547	0.90 (0.63-1.28)	0.572	1.47 (0.91-2.39)	0.087
Model II OR (95%CI)	1.08 (0.79-1.48)	0.629	0.91 (0.61-1.35)	0.693	1.41 (0.81-2.43)	0.163
Model III OR (95%CI)	1.11 (0.80-1.55)	0.511	0.99 (0.65-1.51)	0.976	1.27 (0.71-2.28)	0.342
Green pepper						
Model I OR (95%CI)	0.85 (0.64-1.14)	0.252	0.78 (0.54-1.12)	0.141	0.99 (0.60-1.62)	0.894
Model II OR (95%CI)	0.87 (0.64-1.20)	0.328	0.74 (0.49-1.12)	0.090	0.99 (0.57-1.72)	0.973
Model III OR (95%CI)	0.81 (0.57-1.13)	0.167	0.67 (0.44-1.04)	0.037	0.93 (0.51-1.68)	0.844
Radish						
Model I OR (95%CI)	0.97 (0.72-1.31)	0.599	0.89 (0.62-1.28)	0.468	1.22 (0.72-2.06)	0.870
Model II OR (95%CI)	0.92 (0.67-1.28)	0.348	0.90 (0.60-1.35)	0.489	1.16 (0.65-2.07)	0.799
Model III OR (95%CI)	0.98 (0.69-1.39)	0.495	0.92 (0.60-1.43)	0.578	1.27 (0.68-2.35)	0.893
Spinach						
Model I OR (95%CI)	0.77 (0.58-1.03)	0.173	0.62 (0.44-0.90)	0.024	0.93 (0.58-1.51)	0.923
Model II OR (95%CI)	0.80 (0.58-1.09)	0.283	0.66 (0.45-0.99)	0.071	1.02 (0.59-1.77)	0.851
Model III OR (95%CI)	0.86 (0.61-1.20)	0.532	0.78 (0.51-1.20)	0.360	0.94 (0.52-1.70)	0.821
Radish kimchi						
Model I OR (95%CI)	0.82 (0.62-1.10)	0.193	0.69 (0.48-0.99)	0.038	1.33 (0.80-2.22)	0.592
Model II OR (95%CI)	0.82 (0.59-1.12)	0.195	0.70 (0.47-1.05)	0.090	1.21 (0.69-2.11)	0.937
Model III OR (95%CI)	0.80 (0.57-1.12)	0.192	0.73 (0.47-1.13)	0.142	1.06 (0.58-1.94)	0.816

Cabbage						
Model I OR (95%CI)	0.34 (0.25-0.46)	< 0.001	0.37 (0.26-0.53)	< 0.001	0.33 (0.19-0.55)	< 0.001
Model II OR (95%CI)	0.45 (0.32-0.63)	< 0.001	0.50 (0.34-0.75)	0.004	0.45 (0.25-0.81)	0.016
Model III OR (95%CI)	0.50 (0.35-0.72)	0.001	0.53 (0.35-0.82)	0.015	0.54 (0.29-1.00)	0.094
Chonggak kimchi						
Model I OR (95%CI)	0.83 (0.62-1.10)	0.215	0.69 (0.48-0.99)	0.038	1.33 (0.80-2.20)	0.589
Model II OR (95%CI)	0.83 (0.60-1.04)	0.253	0.69 (0.46-1.04)	0.077	1.21 (0.69-2.11)	0.933
Model III OR (95%CI)	0.81 (0.58-1.13)	0.244	0.72 (0.47-1.11)	0.113	1.06 (0.58-1.94)	0.818
Zucchini						
Model I OR (95%CI)	1.01 (0.76-1.35)	0.898	0.96 (0.67-1.37)	0.772	1.38 (0.84-2.25)	0.195
Model II OR (95%CI)	1.09 (0.79-1.51)	0.783	0.99 (0.66-1.48)	0.846	1.87 (1.06-3.28)	0.026
Model III OR (95%CI)	1.11 (0.78-1.56)	0.749	1.97 (0.63-1.50)	0.784	1.82 (1.00-3.30)	0.045
Chinese cabbage						
Model I OR (95%CI)	0.64 (0.47-0.86)	< 0.001	0.53(0.36-0.78)	< 0.001	0.80 (0.49-1.31)	0.342
Model II OR (95%CI)	0.58 (0.41-0.81)	< 0.001	0.49(0.32-0.76)	< 0.001	0.72 (0.41-1.25)	0.115
Model III OR (95%CI)	0.62 (0.44-0.89)	< 0.001	0.57(0.36-0.90)	0.005	0.67 (0.37-1.22)	0.092
Lettuce						
Model I OR (95%CI)	0.64 (0.48-0.86)	0.008	0.58 (0.41-0.82)	0.013	0.77 (0.47-1.26)	0.301
Model II OR (95%CI)	0.67 (0.49-0.93)	0.026	0.58 (0.39-0.86)	0.023	0.79 (0.45-1.36)	0.365
Model III OR (95%CI)	0.68 (0.48-0.95)	0.031	0.58 (0.38-0.88)	0.023	0.78 (0.43-1.40)	0.337
Onion						
Model I OR (95%CI)	1.06 (0.79-1.42)	0.817	0.90 (0.62-1.30)	0.436	1.21 (0.73-1.99)	0.539
Model II OR (95%CI)	1.09 (0.79-1.51)	0.693	0.84 (0.56-1.27)	0.320	1.33 (0.76-2.33)	0.344
Model III OR (95%CI)	1.13 (0.80-1.59)	0.572	0.90 (0.58-1.40)	0.524	1.27 (0.71-2.30)	0.457
Mustard leaf Kimchi						
Model I OR (95%CI)	0.84 (0.62-1.12)	0.099	0.87 (0.60-1.25)	0.220	0.66 (0.40-1.08)	0.207
Model II OR (95%CI)	0.76 (0.55-1.06)	0.018	0.84 (0.56-1.27)	0.089	0.60 (0.34-1.04)	0.076
Model III OR (95%CI)	0.76 (0.54-1.08)	0.038	0.90 (0.58-1.40)	0.180	0.57 (0.32-1.04)	0.093
Green onion						
Model I OR (95%CI)	1.03 (0.76-1.38)	0.909	0.92 (0.64-1.33)	0.527	1.21 (0.73-2.01)	0.612
Model II OR (95%CI)	1.02 (0.73-1.41)	0.807	0.94 (0.62-1.42)	0.588	1.11 (0.64-1.95)	0.731
Model III OR (95%CI)	1.01 (0.71-1.44)	0.744	0.94 (0.60-1.46)	0.582	1.18 (0.65-2.13)	0.650
Mandarins						
Model I OR (95%CI)	0.60 (0.44-0.80)	0.001	0.79 (0.55-1.12)	0.356	0.42 (0.25-0.71)	0.002
Model II OR (95%CI)	0.74 (0.53-1.04)	0.061	0.97 (0.66-1.44)	0.941	0.60 (0.34-1.07)	0.101
Model III OR (95%CI)	0.71 (0.50-1.01)	0.038	0.95 (0.62-1.44)	0.961	0.54 (0.29-0.99)	0.061
Strawberries						
Model I OR (95%CI)	0.44 (0.33-0.60)	< 0.001	0.40 (0.27-0.58)	< 0.001	0.39 (0.23-0.67)	0.001
Model II OR (95%CI)	0.56 (0.40-0.78)	0.001	0.49 (0.32-0.74)	0.001	0.52 (0.29-0.93)	0.026
Model III OR (95%CI)	0.61 (0.43-0.86)	0.009	0.50 (0.32-0.79)	0.004	0.57 (0.30-1.07)	0.065
Orange juice						
Model I OR (95%CI)	0.43 (0.32-0.59)	< 0.001	0.36 (0.25-0.53)	< 0.001	0.50 (0.30-0.85)	0.006
Model II OR (95%CI)	0.61 (0.44-0.85)	0.003	0.47 (0.30-0.71)	0.001	0.83 (0.46-1.51)	0.294
Model III OR (95%CI)	0.65 (0.46-0.94)	0.014	0.49 (0.31-0.77)	0.002	0.98 (0.52-1.86)	0.677
Watermelon						
Model I OR (95%CI)	0.59 (0.44-0.78)	0.003	0.61 (0.43-0.87)	0.032	0.63 (0.39-1.02)	0.117
Model II OR (95%CI)	0.69 (0.50-0.95)	0.065	0.71 (0.48-1.06)	0.211	0.72 (0.42-1.24)	0.309
Model III OR (95%CI)	0.65 (0.46-0.92)	0.043	0.67 (0.44-1.03)	0.132	0.71 (0.40-1.27)	0.292
Apples						
Model I OR (95%CI)	0.40 (0.29-0.54)	< 0.001	0.38 (0.26-0.55)	< 0.001	0.43 (0.26-0.71)	0.005
Model II OR (95%CI)	0.60 (0.43-0.85)	0.006	0.57 (0.37-0.87)	0.026	0.64 (0.37-1.11)	0.204
Model III OR (95%CI)	0.64 (0.45-0.92)	0.028	0.53 (0.34-0.83)	0.013	0.82 (0.46-1.47)	0.705
Persimmons						
Model I OR (95%CI)	0.49 (0.36-0.66)	< 0.001	0.62 (0.43-0.89)	0.026	0.40 (0.24-0.66)	0.002
Model II OR (95%CI)	0.56 (0.40-0.78)	0.001	0.72 (0.48-1.08)	0.151	0.46 (0.26-0.80)	0.018
Model III OR (95%CI)	0.55 (0.38-0.78)	0.001	0.67 (0.44-1.03)	0.086	0.45 (0.25-0.82)	0.028
Bananas						
Model I OR (95%CI)	0.32 (0.24-0.44)	< 0.001	0.33 (0.22-0.47)	< 0.001	0.26 (0.15-0.46)	< 0.001
Model II OR (95%CI)	0.40 (0.29-0.57)	< 0.001	0.41 (0.27-0.62)	< 0.001	0.34 (0.19-0.63)	0.001
Model III OR (95%CI)	0.44 (0.31-0.63)	< 0.001	0.41 (0.27-0.64)	0.001	0.44 (0.23-0.83)	0.014
Citrus tea						
Model I OR (95%CI)	0.64 (0.48-0.87)	0.002	0.56 (0.38-0.81)	0.001	0.81 (0.49-1.34)	0.281
Model II OR (95%CI)	0.78 (0.56-1.09)	0.048	0.68 (0.44-1.04)	0.017	1.00 (0.57-1.76)	0.669
Model III OR (95%CI)	0.83 (0.59-1.18)	0.161	0.71 (0.45-1.11)	0.040	1.14 (0.63-2.09)	0.992

¹OR for the association with the lowest tertile group compared with the highest tertile group; ²Trends were calculated using the median intake for each category of vitamin C-contributing food consumption as a continuous variable: Model I : Unadjusted; Model II : Adjusted by first-degree family history of gastric cancer, education level, job, household income, smoking status, regular exercise; Model III: Additionally adjusted for *H. pylori* infection.

Inverse associations between cabbage, strawberry, and banana consumption and gastric cancer risk were also observed for both men and women. Some different protective factors were found between genders. Starches, potatoes, sweet potatoes, spinach, Chinese cabbage, lettuce, orange juice, and apples decreased the risk of gastric cancer in men, and fruits and persimmons decreased the risk of gastric cancer in women. In particular, zucchini consumption increased the gastric cancer risk in women [1.87 (1.06-3.28)].

DISCUSSION

In our study, we found a negative association between vitamin C intake and gastric cancer in the crude model and the adjusted model. The association became less apparent after an additional adjustment for *H. pylori* status. After adjustment for confounders, vitamin C intake showed no protective effect for participants infected with *H. pylori*. The consumption of cabbage, strawberries, and bananas had inverse associations with gastric cancer risk based on the results of the overall adjusted model and for both genders.

The association between vitamin C intake and the risk of gastric cancer is supported by many observational and meta-analysis studies. In a meta-analysis of 11 observational studies, a dose-response analysis was conducted for vitamin C intake (100 mg/d), which showed a significant reduction in the risk of gastric cancer [RR (95%CI): 0.74 (0.69-0.79)]^[6]. An inverse association between the intake of vitamin C and the risk of gastric cancer was consistent among case-control studies^[7-13] and cohort studies^[14,15]. For example, in a Spanish study, the strongest protective effects were observed for vitamin C from fruits and vegetables^[12]. Another case-control study in Italy reported that increased vitamin C consumption exhibited an inverse relationship to the risk of gastric cancer^[13]. Our result is consistent with a cohort study from Netherlands that reported that an inverse association between vitamin C and the risk of gastric carcinoma was found in age- and gender-adjusted analyses. However, this association became weaker and was of borderline significance in the multivariate analysis (which included age, gender, smoking history, education, stomach disorders, and family history of gastric cancer) [RR (95%CI): 0.70 (0.50-1.00)]^[14]. Therefore, it appears that vitamin C is among the most consistent protective factors against gastric carcinogenesis. This protective effect may be related to the antioxidant effects of vitamin C, free radical scavenger effects, and the inhibition of nitrosamine formation^[30,31]. Another biological explanation for the inverse association is the direct action of vitamin C on the growth of *H. pylori*^[32]. However, no clear protective effect of vitamin C intake was observed in participants infected with *H. pylori* in our study.

In contrast, some observational studies did not successfully demonstrate a significant association

between vitamin C intake and gastric cancer. Two case-control studies conducted in Mexico and Italy that included a small number of participants, showed no protective effect of vitamin C^[16,17]. The Shanghai Women's and Men's Health study showed that none of the dietary nutrients examined, including vitamin A, vitamin C, vitamin E, carotene, retinol, selenium, or folic acid, were associated with the distal gastric cancer risk among men or women^[18].

In the present study, we failed to find a protective effect of vitamin C against gastric cancer in participants infected with *H. pylori*. At least three explanations for this finding should be considered. First, the consumption of fruits and vegetables, which are the main sources of vitamin C, is highly prevalent among the Korean population^[33]. In our study, a difference between case and control groups was observed only for total fruit consumption. Therefore, if an association between vitamin C intake and gastric cancer truly exists, the small difference between the case and control groups in our study may have limited the statistical power to detect this association. Second, this finding may be related to the Korean habit of eating pickled or processed vegetables, which includes many types of kimchi. Kimchi is a fermented vegetable with a high concentration of salt and pepper, which are important risk factors for gastric cancer^[3]. Moreover, a high dietary salt intake can exacerbate *H. pylori* infection in gastric cancer patients^[34]. Therefore, it is not surprising that no difference in vegetable consumption was observed between the case and control groups, which may weaken the protective effect of vitamin C. Additionally, the exacerbating role of *H. pylori* infection may modify the true association between vitamin C intake and gastric cancer risk in the adjusted model. Therefore, the protective effect of vitamin C should be considered in the model without adjusting for *H. pylori* status. Finally, the amount of vitamin C consumed by the participants with *H. pylori* infection could explain this finding. A Korean case-control study reported that consuming over 170 mg/d of vitamin C could protect people with *H. pylori* infection against the risk of gastric cancer [0.10 (0.02-0.63)]^[10]. Hence, in our analysis, vitamin C doses of 120 mg/d may not be high enough to show protective effect of vitamin C in participants infected with *H. pylori*.

In the vitamin C-contributing food consumption analyses, our findings are consistent with a meta-analysis of prospective cohort studies that reported an inverse association between fruit intake and gastric cancer incidence [RR (95%CI): 0.82 (0.73-0.93)] that was stronger for follow-up periods of ≥ 10 years [0.66 (0.52-0.83)]; however, no such association was observed for vegetable consumption [0.88 (0.69-1.13)]^[35]. Another meta-analysis of 8 observational studies of Korean and Japanese populations also showed that an increased intake of fresh vegetables was significantly associated with a decreased risk of gastric cancer

[OR (95%CI): 0.62 (0.46-0.85)]^[36]. Other meta-analyses of observational studies have supported the protective effect of fruits and vegetables against gastric cancer^[37-40]. Additionally, the protective effect of fruits and vegetables has been consistently reported in many other case-control studies^[8,11,41-46] and prospective cohort studies^[47-50]. However, some cohort studies did not find this association^[51-54]. For example, our findings are inconsistent with a cohort study from Japan that reported non-significant associations for the consumption between fruit and vegetable consumption and gastric cancer incidence^[54]. This finding is comparable with the results of the Netherlands Cohort Study, which showed inverse associations between gastric cancer and the consumption of total vegetables, pulses, raw leaf vegetables, total fruits, citrus fruits, and apples and pears in the crude analysis that became weaker or disappeared in the multivariate analysis^[51].

The methods used to cook fruits and vegetables may play an important role in the relationship between fruit and vegetable consumption and gastric cancer risk. Some studies have reported that an increased consumption of pickled or processed vegetables increases the risk of gastric cancer^[36,46,55-57]. A meta-analysis of 14 observational studies demonstrated that an increased intake of pickled vegetables was significantly associated with an increased risk of gastric cancer [OR (95%CI): 1.28 (1.06-1.53)]^[36]. Moreover, a Korean study reported that increased intake of salt-fermented fish and kimchi was associated with an elevated risk of early gastric cancer^[46]. These findings explain the non-significant associations in our study because Koreans frequently consume processed vegetables, such as cooked, salted, or pickled vegetables, instead of fresh vegetables, and these often include a high concentration of salt. This increased salt consumption could weaken the protective effect of vegetables against gastric cancer.

Some strengths of the present study include the use of a comprehensive, validated FFQ to assess of the exposure to factors of interest. Additionally, we collected information from the participants about the prevalence of *H. pylori* infection, which an IARC monograph names as a cause of stomach cancer^[19,20].

However, some potential limitations are also present in our hospital-based case-control study, such as selection and recall bias. Selection bias occurs in a case-control study when subjects in the "control" group are not truly representative of the population that is included in the case group. The hospital-based control group may not represent the Korean population. Moreover, the small number of participants in our study may not be sufficient to detect the protective effects of vitamin C and vitamin C-contributing foods on the gastric cancer risk. Finally, subgroup analyses by anatomical site (cardia vs non-cardia) or histological type (intestinal vs diffuse) would be helpful because these factors may modify the epidemiological characteristics of gastric cancer.

In conclusion, an inverse association was found between vitamin C and the risk of gastric cancer. Sufficient evidence is lacking to support the protective effect of vitamin C intake in participants infected with *H. pylori*. The total fruit consumption and some vitamin C-contributing foods showed a negative association with gastric cancer. Further studies that replicate our results in larger sample are required.

COMMENTS

Background

Vitamin C is one of the most common antioxidants in fruits and vegetables and it may exert a chemopreventive effect. However, the association between vitamin C intake and gastric cancer risk has been inconsistent among epidemiological studies.

Research frontiers

The authors conducted a case-control study to investigate the association between vitamin C, foods containing vitamin C consumption and gastric cancer risk.

Innovations and breakthroughs

Protective effect of vitamin C and some vitamin C-contributing foods against gastric cancer risk was observed in this study. Additionally, the authors collected information from the participants about the prevalence of *H. pylori* infection, which an IARC monograph names as a cause of stomach cancer. However, they failed to find a protective effect of vitamin C against gastric cancer in participants infected with *H. pylori*.

Applications

Results of this study support for using vitamin C and some vitamin C-contributing foods to protect people against gastric cancer risk.

Terminology

Dietary vitamin C intake has a chemopreventive effect, which may reduce gastric cancer risk. The normal metabolism in human body or exposure to well-known carcinogenesis can produce reactive oxygen species. At a cellular level, these species cause various mutations and other consequences in the DNA. Vitamin C plays a role in blocking carcinogenesis to protect cells from this damage and development of gastric cancer.

Peer-review

The manuscript by Hoang and colleagues describes the risk of gastric cancer as a function of vitamin C intake, an epidemiological study involving more than 1200 cases who participated in the study through questionnaires and other tests. The study is well performed, and the manuscript is written in good and logical order easy for the reader to digest.

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Retrospective Study

Feasibility and safety of endoscopic submucosal dissection for lower rectal tumors with hemorrhoids

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Author contributions: Tanaka S designed and performed the research and wrote the paper; Toyonaga T designed the research and supervised the report; Ohara Y, Yoshizaki T and Kawara F designed the research and contributed to the analysis; Morita Y, Hoshi N and Ishida T provided clinical advice; Umegaki E and Azuma T supervised the report.

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

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of Kobe University Hospital.

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Abstract

AIM: To evaluate the feasibility and safety of endoscopic submucosal dissection (ESD) for lower rectal lesions with hemorrhoids.

METHODS: The outcome of ESD for 23 lesions with hemorrhoids (hemorrhoid group) was compared with that of 48 lesions without hemorrhoids extending to the dentate line (non-hemorrhoid group) during the same study period.

RESULTS: Median operation times (ranges) in the hemorrhoid and non-hemorrhoid groups were 121 (51-390) and 130 (28-540) min. The *en bloc* resection rate and the curative resection rate in the hemorrhoid group were 96% and 83%, and they were 100% and 90% in the non-hemorrhoid group, respectively. In terms of adverse events, perforation and postoperative bleeding did not occur in both groups. In terms of the clinical course of hemorrhoids after ESD, the rate of complete recovery of hemorrhoids after ESD in lesions with resection of more than 90% was significantly higher than that in lesions with resection of less than 90%.

CONCLUSION: ESD on lower rectal lesions with hemorrhoids could be performed safely, similarly to that on rectal lesions extending to the dentate line without hemorrhoids. In addition, all hemorrhoids after ESD improved to various degrees, depending on the resection range.

Key words: Endoscopic submucosal dissection; Rectum; Hemorrhoid; Outcome; Bleeding

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Core tip: Recently, the feasibility and safety of endoscopic submucosal dissection (ESD) have been reported from different countries. However, ESD for lesions extending to the dentate line is technically difficult due to the anatomical features. This paper showed ESD on lower rectal lesions with hemorrhoids was feasible and safe, similarly to that on rectal lesions extending to the dentate line without hemorrhoids and all hemorrhoids after ESD improved to various degrees, depending on the resection range.

Tanaka S, Toyonaga T, Morita Y, Hoshi N, Ishida T, Ohara Y, Yoshizaki T, Kawara F, Umegaki E, Azuma T. Feasibility and safety of endoscopic submucosal dissection for lower rectal tumors with hemorrhoids. *World J Gastroenterol* 2016; 22(27): 6268-6275 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6268.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6268>

INTRODUCTION

Endoscopic submucosal dissection (ESD) is a standard therapy for gastric neoplasms. Recently, ESD has been applied for superficial colorectal neoplasms and the number of publications about it has been increasing^[1-3]. In addition, the feasibility and safety of this procedure have been reported from both Asia and Western countries^[4,5]. However, ESD for lesions extending to the dentate line is technically difficult because these lesions sometimes have severe fibrosis^[6], and the lumen is narrow due to the anal sphincter. Moreover, in this area, there are often coexisting hemorrhoids. For lesions with hemorrhoids, the maintenance of a good endoscopic view is not easy, and the management of large hemorrhoid vessels makes ESD increasingly difficult. However, ESD for lower rectal lesions should be considered if possible because surgery can result in a permanent colostomy or rectal dysfunction, and the patients' quality of life is tremendously affected. To the best of our knowledge, the feasibility of ESD for rectal lesions with hemorrhoids has not been fully studied. In this study, we aimed to evaluate the feasibility and safety of ESD for lower rectal lesions with hemorrhoids and the clinical course of hemorrhoids thereafter.

MATERIALS AND METHODS

Patients

A total of 1485 colorectal neoplasms in 1357 patients were treated with ESD at Kobe University Hospital and Kishiwada Tokushukai Hospital between April 2005 and May 2014. The inclusion criteria for colorectal ESD were (1) lesions over 20 mm in size; (2) lesions with scars due to previous endoscopic treatment or biopsies; (3) local recurrent lesion after previous endoscopic or surgical resection; and (4) invasive carcinoma with slight submucosal invasion (< 1000 μ m from the muscularis mucosa) estimated by conventional endoscopic and magnification chromoendoscopic examinations according to previous reports^[7-9]. Among these lesions, 23 lesions coexisted with hemorrhoids. The presence of hemorrhoids was defined as the endoscopic finding that vessels in the area around the anus and rectum were clearly swollen and elevated above the surrounding mucosal level. All hemorrhoids diagnosis was performed by two endoscopists (Tanaka S and Ohara Y). When their diagnosis was different, it was confirmed afterward by two endoscopists. The outcome of ESD for the lesions with hemorrhoids (hemorrhoid group) was compared with that of 48 lesions without hemorrhoids extending to the dentate line (non-hemorrhoid group) during the same study period. The positional relationship between the lesion and the dentate line was confirmed using an endoscope. The size of the lesion was confirmed by pathological assessment. The morphological types of colorectal lesions were classified as lateral spreading tumor granular type (LST-G), lateral spreading tumor non-granular type (LST-NG), protruding type and depressed type. This is a retrospective study, and we retrospectively reviewed the medical records including patient characteristics, details of endoscopic treatment and pathological features of the resected lesions.

ESD setting

Four endoscopists carried out the procedure. They were all well-trained endoscopists with substantial experience of ESD (more than 100 cases). ESD was carried out with Flush knife (DK2618JN10; FUJIFILM) or Flush knife BT (DK-2618JN; FUJIFILM) through a conventional single-channel endoscope (CF-240I, CF-260AI, GIF-Q260J; Olympus Medical Systems). The Flush knife and Flush knife BT used were 1.5 mm in length. A transparent hood (D-201-11084; Olympus, 16675; TOP) was mounted on the tip of the endoscope to maintain a clear operative field. An ICC 200 or VIO 300D (ERBE Elektromedizin, GmbH, Tübingen) was used as the power source for electrical cutting and coagulation. The procedure time was measured from the first submucosal saline injection to lifting of the target lesion to the end of the procedure (when *en bloc* resection was achieved), using a stopwatch. The procedure speed, the area of mucosa dissected per

Table 1 Patient and lesion characteristics

	Hemo group	Non-Hemo group	P value
Patients	23	48	
Lesions	23	48	
Sex			
Male	9	17	0.9
Female	14	31	
Age (yr), median (range)	69 (48-79)	65 (40-87)	0.4
Morphological type			
LST-G	17	34	1.0
LST-NG	0	1	
Protruding	6	13	
Tumor size (mm), median (range)	53 (6-158)	52 (7-155)	0.81
Specimen size (mm), median (range)	65 (28-165)	62 (25-180)	0.9
Histology			
Adenoma	7	9	0.84
Tis-T1a	15	34	
T1b or deeper	1	5	

minute (mm²/min), was calculated by the area of the resected specimen divided by the procedure time. The approximate oval area (mm²) of the resected specimen was calculated as follows: $3.14 \times 0.25 \times \text{long axis} \times \text{minor axis}$ ^[10].

ESD technique

Submucosal injection: There are sensory nerves in the squamous epithelium below the dentate line. Thus, when submucosal injection was performed at the anal side, to prevent pain, 1% lidocaine (100 mg/10 mL) diluted 1:1 with hyaluronic acid solution was used as submucosal injection solution.

Mucosal incision: To avoid bleeding, a shallow mucosal incision was performed and the vessels were exposed while inflicting as little damage as possible (Figure 1A-C). The exposed vessels were coagulated using hemostatic forceps.

Submucosal dissection: At the anal canal area, the submucosal layer is connected tightly with mucosal epithelium by submucosal muscle strands (musculus submucosa ani), which is derived from longitudinal muscle of rectum (Figure 1D). Dissociation of this submucosal muscle strands completely and reaching just above the muscularis propria layer are very important (Figure 1E). The submucosal dissection was performed just above the muscularis propria layer and the penetrating vessels were handled using an electro-knife itself or hemostatic forceps (Figure 1F-H).

Adverse events

Postoperative bleeding was defined as: (1) requiring endoscopic hemostatic treatment; (2) when the total hemoglobin dropped by more than 2 g/dL compared

with the last preoperative level; or (3) cases of massive melena after ESD with no other apparent source of bleeding. Perforation was diagnosed by endoscopic findings during the endoscopic treatment, or was diagnosed by the presence of free air on abdominal plain radiography or computed tomography

Ethics

All patients were informed of the risks and benefits of ESD and provided written informed consent. The study protocol was approved by the ethics committee of Kobe University Hospital and Kishiwada Tokushukai Hospital.

Statistical analysis

Statistical analyses were conducted using SPSS Statistics 18.0 (SPSS, Chicago, IL, United States). Proportions of categorical variables were compared using two-sided Fisher's exact test and χ^2 test. Continuously distributed variables were compared using Student's *t*-test, and non-continuous variables were assessed using Wilcoxon's rank-sum test. Values of *P* < 0.05 were considered significant.

RESULTS

Characteristics of the patients and resected lesions

In this study, 23 lesions were classified into the hemorrhoid group and 48 into the non-hemorrhoid group. Characteristics of the patients and the lesions are shown in Table 1. The median tumor size (range) in the hemorrhoid group was 53 (6-158) mm and that in the non-hemorrhoid group was 52 (7-155) mm. Histological examination revealed that there were 7 cases of adenoma, 15 of Tis-T1b cancer and 1 of T1b or deeper cancer in the hemorrhoid group, and 9 cases of adenoma, 34 of Tis-T1a and 5 of T1b or deeper cancer in the non-hemorrhoid group. These data showed no significant difference between the two groups.

Outcomes and adverse events

Table 2 shows a summary of the outcomes and adverse events of the treated lesions. The median operation time (range) in the hemorrhoid group was 121 (51-390) min, and it was 130 (28-540) min in the non-hemorrhoid group, which were not significantly different. Median procedure speed (range) in the hemorrhoid group was 0.18 (0.05-0.47) mm²/min, and it was 0.20 (0.02-0.57) mm²/min in the non-hemorrhoid group. These results were not significantly different.

The *en bloc* resection rate and the curative resection rate in the hemorrhoid group were 96% and 83%, and they were 100% and 90% in the non-hemorrhoid group, respectively. These rates were not significantly different between the two groups. In terms of adverse events, perforation and postoperative

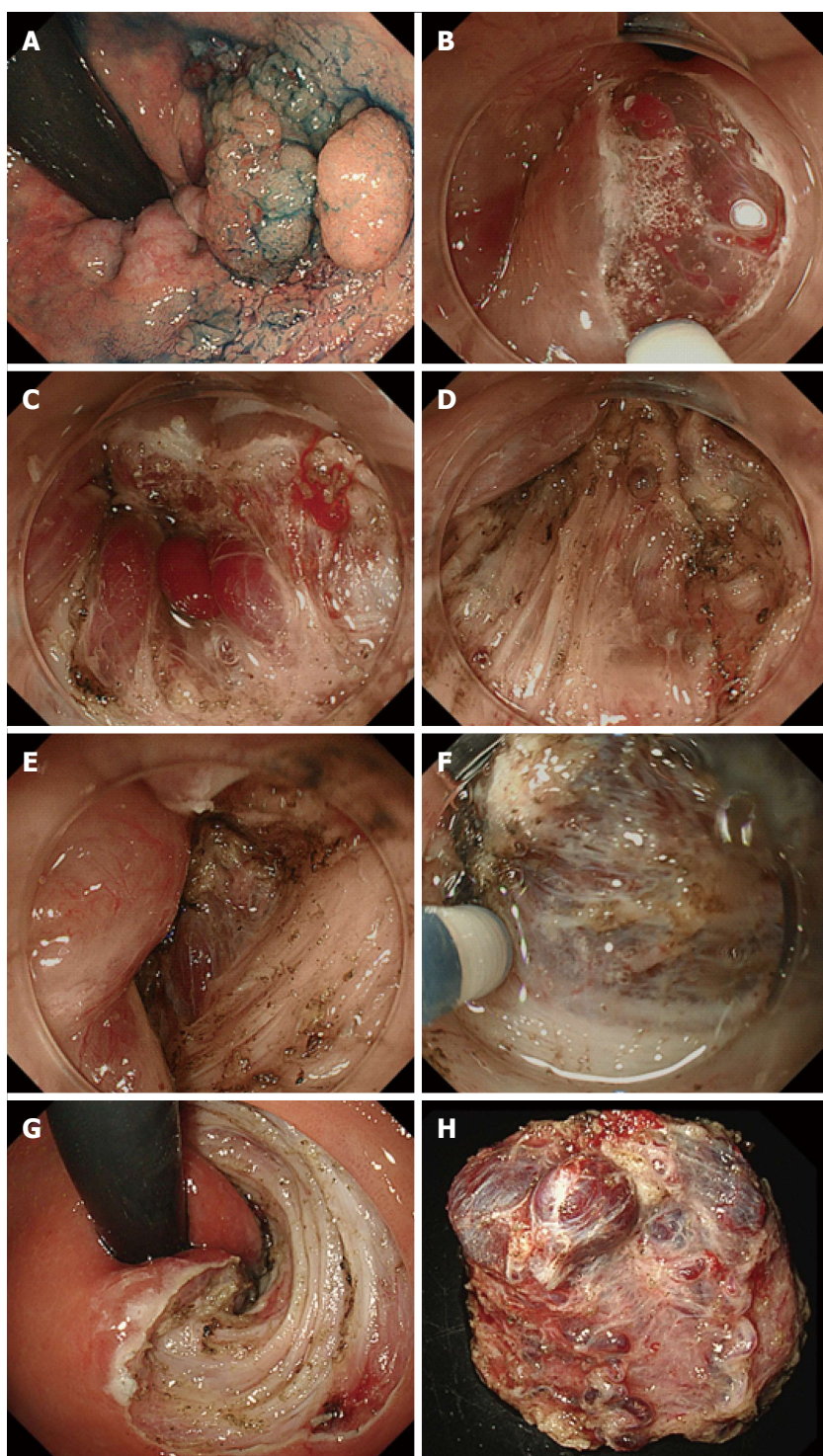


Figure 1 Endoscopic submucosal dissection for lower rectal lesion with hemorrhoids. A: The laterally spreading tumor granular type (nodular mixed type) with hemorrhoids located in the lower rectum and expanding to half of the lumen; B: A shallow mucosal incision was performed and the vessels were exposed while inflicting as little damage as possible; C: The exposed vessels were coagulated using hemostatic forceps; D: The submucosal layer is tightly connected with submucosal muscle strands; E: Dissociation of submucosal muscle strands completely; F: The submucosal dissection was performed just above the muscularis propria layer; G: The ulcer floor after ESD; H: The resected specimen with many vessels.

bleeding did not occur in both groups.

Of the 23 patients, in 21 patients, the result was curative resection. Of the 2 patients with non-curative resection, both underwent surgical treatment. After surgical resection, neither case had recurrence during a median follow-up period of 27.5 mo (range 12-43

mo). Of the 21 patients with curative resection, 3 patients did not receive follow-up colonoscopy for personal reasons, and 18 patients underwent surveillance colonoscopy. Among them, there were no cases of recurrence during a median follow-up period of 36 mo (range 2-99 mo) (Figure 2).

Table 2 Results of endoscopic submucosal dissection *n* (%)

	Hemo group	Non-Hemo group	<i>P</i> value
Operation time (min), median (range)	121 (51-390)	130 (28-540)	0.98
Procedure speed (mm ² /min), median (range)	0.18 (0.05-0.47)	0.20 (0.02-0.57)	0.46
<i>En bloc</i> resection	22 (96)	48 (100)	0.32
Curative resection	19 (83)	43 (90)	0.32
Adverse events			
Perforation	0 (0)	0 (0)	1.00
Postoperative bleeding	0 (0)	0 (0)	1.00

Clinical course of hemorrhoids after ESD

Among the 23 lesions with hemorrhoids, 22 lesions were classified into Goligher classification type 1 and 1 lesion into Goligher classification type 2. There were no patients in Goligher classification type 3 or 4. In terms of the clinical course of hemorrhoids after ESD, among 13 lesions with resection of less than 90% of the circumference, 11 lesions partially regressed (Figure 3) and complete recovery was achieved for 2 lesions (Figure 4). Among 5 lesions with resection of more than 90% including 2 lesions resected circumferentially, complete recovery of all hemorrhoids was achieved. The rate of complete recovery of hemorrhoids after ESD in lesions with resection of more than 90% was significantly higher than that in lesions with resection of less than 90% ($P < 0.01$). One patient with Goligher classification type 2 had complained anus pain from hemorrhoids, but the symptom completely disappeared after ESD. There were no lesions for which the hemorrhoids worsened.

DISCUSSION

Lower rectal lesions extending to the dentate line are considered difficult to remove endoscopically because of the narrow lumen, the risk of bleeding from the rectal venous plexus, and anal pain through sensory nerves in the anoderm^[6]. The recent development of devices and techniques for ESD has allowed the performance of *en bloc* resection of technically difficult lesions^[10-13]. Several reports have described the safety and efficacy of ESD of rectal lesions extending to the dentate line^[6,14]. However, lower rectal lesions with hemorrhoids seemed to be technically more difficult. In lower rectal lesions, the quality of life (QOL) of patients is greatly affected by the treatment method. Surgery is preferably avoided in terms of the patient's QOL after treatment due to the risk of a permanent colostomy and rectal dysfunction. Transanal resection (TAR) for lower rectal tumors is useful with few complications; however, local recurrence has been reported in 23% to 31% of cases^[15-17]. Kiriya *et al.*^[15] reported ESD to be more effective than TAR for treating noninvasive rectal tumors, with a lower recurrence rate and a

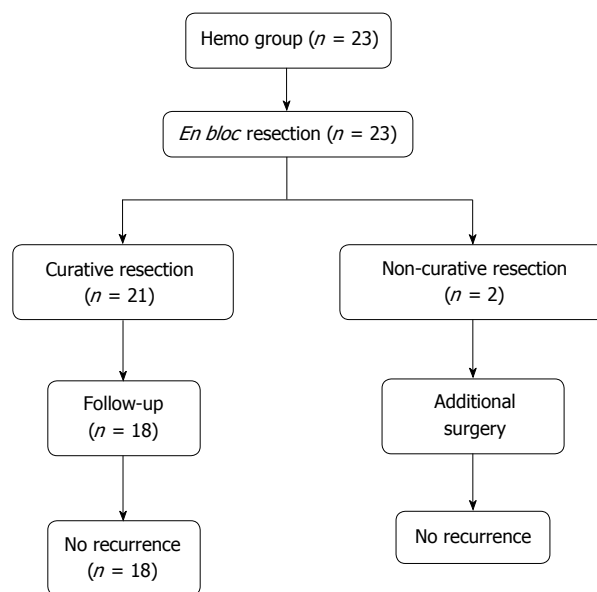


Figure 2 Flow chart of patients who underwent endoscopic submucosal dissection for lower rectal lesions with hemorrhoids.

shorter hospital stay, despite a longer procedure time. Moreover, Nam *et al.*^[18] reported Overall direct medical costs were significantly lower for ESD than for TAR in the treatment of rectal tumors. Therefore, ESD seems to be the most non-invasive and reliable treatment for lower rectal lesions. Furthermore, if ESD could be performed on lower rectal lesions with hemorrhoids, it could be a better treatment in terms of avoiding recurrence and improving patient's QOL.

In previously reported studies, esophageal cancer with esophageal varices could be resected endoscopically by the eradication of varices using endoscopic variceal ligation (EVL) or endoscopic injection sclerotherapy (EIS) before endoscopic resection^[19-22]. Superficial esophageal neoplasms can be removed endoscopically even if they are located on varices. As varices have a steady blood flow from the anal to the oral side, EVL or EIS can be carried out on the anal side away enough from the tumor, so that the secondary shrinkage and fibrosis do not affect the endoscopic tumor resection. However, since the hemorrhoidal plexus is controlled by the superior rectal artery, middle rectal artery and inferior rectal artery, the blood supply is more complicated than for esophageal varices. Sclerotherapy or ligation therapy has to be performed in the proximity of the tumor, and banding or fibrosis after treatment makes ESD more difficult. Thus, ESD for lower rectal lesions with hemorrhoids has to be carried out without pre-treatment.

In previous reports, technical points of ESD for lower rectal lesions extending to the dentate line have been described as follows: (1) local anesthesia to prevent anal pain before the submucosal injection of hyaluronic acid; (2) a shallow peripheral mucosal incision to prevent bleeding; and (3) appropriate

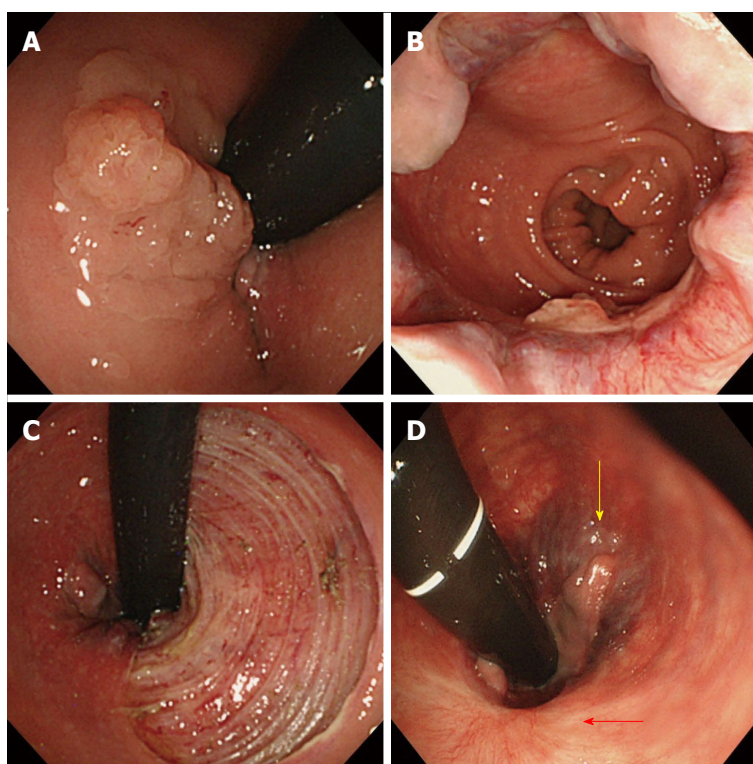


Figure 3 Endoscopic submucosal dissection (ESD) for lower rectal lesions with hemorrhoids. A, B: The laterally spreading tumor granular type (nodular mixed type) with hemorrhoids located at the lower rectum expanding to half of the lumen; C: The ulcer floor after endoscopic submucosal dissection (ESD); D: Hemorrhoid was partially improved (1 year after ESD). Yellow arrow indicates remnant hemorrhoids and red arrow indicates ESD scar.

handling of the blood vessels with hemostatic forceps^[6]. The strategy of ESD for lower rectal lesions with hemorrhoids is almost the same as that described above in our institute. However, we pay particular attention to the depth of mucosal incision and the appropriate level of submucosal dissection. The creation of sufficient space between mucosa and submucosal vessel by injecting hyaluronic acid solution and the performance of a shallow mucosal incision without hemorrhoid injury are necessary to prevent bleeding. The submucosal layer at the anal canal is tightly connected to the mucosal epithelium. Cutting the submucosal layer completely and reaching just above the muscularis propria are important. In addition, hemorrhoid vessels penetrate the muscle layer vertically and hemorrhoids develop at the level of the middle submucosal layer. If the submucosal dissection is performed at the level just above the muscularis propria layer, the only penetrating vessels have to be processed and the source of blood supply into hemorrhoids could be shut off. If the dissecting level is too shallow or kept at the middle submucosal layer, many hemorrhoid vessels would be exposed and it would require a substantial amount of time to process them. In addition, if complete handling of vessels to shut off the blood flow were not performed, hemorrhoids might not be improved after ESD. Therefore, the maintenance of an appropriate dissection level is important to perform ESD safely and effectively, not only to remove the tumor, but also to make the hemorrhoids disappear.

By following the technical tips, a higher rate of *en bloc* resection without adverse events was achieved in this study.

There were no cases in which hemorrhoids worsened after ESD. All hemorrhoids after ESD were improved to various degrees, depending on the resection range. In all cases that required resection of more than 90% of the circumference, the hemorrhoids completely disappeared. Even in lesions with resection of less than 90% of the circumference, the hemorrhoids were completely improved in 2 of 13 lesions. One of them had severe hemorrhoids (Goligher classification type 2); however, in this case, complete recovery was achieved by resection of only half the circumference (Figure 4). The effectiveness of ESD for hemorrhoids can be explained by two mechanisms. One is that it allows the cutting and shutting off of blood vessels feeding into the hemorrhoids. The other is fibrosis after ESD, which could have a similar effect to APC after EIS or parasclectomy for esophageal varices.

In terms of the outcomes of ESD in the hemorrhoid group and the non-hemorrhoid group, although the hemorrhoid group seemed to be associated with greater technical difficulty than the non-hemorrhoid group, there were no significant differences in operation time, procedure speed, *en bloc* resection rate and adverse events. This result should not be considered to indicate that the technical difficulty was the same with or without the presence of hemorrhoids. In our study, all ESDs were performed by endoscopists

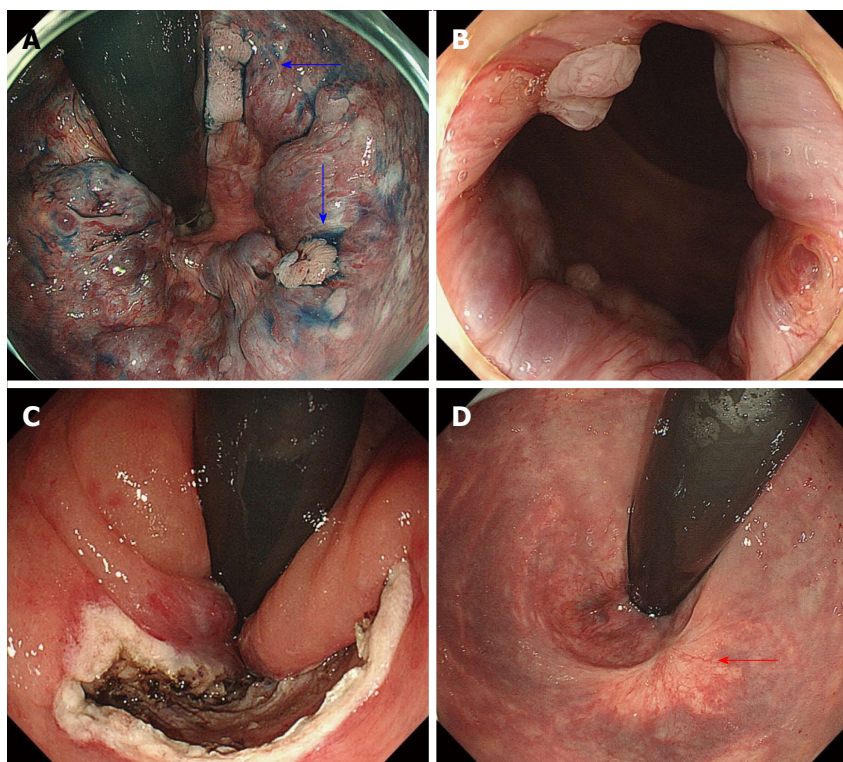


Figure 4 Endoscopic submucosal dissection for lower rectal lesions with hemorrhoids. A, B: Two 0-IIa lesions (blue arrows) located on the hemorrhoids; C: The ulcer floor after ESD; D: Complete recovery from hemorrhoids was achieved (6 mo after ESD). Red arrow indicates ESD scar.

with sufficient experience. It should be noted that ESD is technically difficult and requires adequate experience and advanced skills, so it should be performed by well-trained endoscopists.

There were no cases of recurrence during the follow-up period. This was presumably significantly contributed to by the achievement of a high *en bloc* resection rate. However, six cases could be followed up within only 12 mo and the follow-up periods were thus insufficient, so further investigation is necessary.

In conclusion, ESD on lower rectal lesions with hemorrhoids could be performed safely, similarly to that on rectal lesions extending to the dentate line without hemorrhoids. In addition, all hemorrhoids after ESD could be improved to various degrees depending on the resection range. ESD for lower rectal lesions could be a good indication for lower rectal lesions in spite of the presence of hemorrhoids.

COMMENTS

Background

Recently, the feasibility and safety of endoscopic submucosal dissection (ESD) have been reported from different countries. However, ESD for lesions extending to the dentate line is technically difficult due to the anatomical features. Moreover, if the lesion coexists with hemorrhoids, ESD might be increasingly difficult.

Research frontiers

The prior report described that ESD for lesions extending to the dentate line is technically difficult due to severe fibrosis. In this area, there are often coexisting hemorrhoids. This study evaluated the feasibility of ESD for rectal

lesions with hemorrhoids.

Innovations and breakthroughs

In this study, ESD on lower rectal lesions with hemorrhoids could be performed safely, similarly to that on rectal lesions extending to the dentate line without hemorrhoids. In addition, all hemorrhoids after ESD could be improved to various degrees depending on the resection range.

Applications

This study suggested that ESD for lower rectal lesions could be a good indication for lower rectal lesions in spite of the presence of hemorrhoids.

Peer-review

The author of this paper evaluated that ESD on lower rectal lesions with hemorrhoids could be performed safely, similarly to that on rectal lesions extending to the dentate line without hemorrhoids.

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Observational Study

Favorable lifestyle before diagnosis associated with lower risk of screen-detected advanced colorectal neoplasia

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Informed consent statement: The participants gave their consent to participate in the lifestyle study by completing and returning the mailed questionnaire.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: Statistical code and dataset available from the corresponding author at (t.d.lange@medisin.uio.no) No additional data are available.

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Abstract

AIM: To investigate the association between adherence

to health recommendations and detection of advanced colorectal neoplasia (ACN) in colorectal cancer (CRC) screening.

METHODS: A total of 14832 women and men were invited to CRC screening, 6959 in the fecal immunochemical test arm and 7873 in the flexible sigmoidoscopy arm. These were also sent a self-reported lifestyle questionnaire to be completed prior to their first CRC screening. A lifestyle score was created to reflect current adherence to healthy behaviors in regard to smoking, body mass index, physical activity, alcohol consumption and food consumption, and ranged from zero (poorest) to six (best). Odds ratios (ORs) and 95% CIs were calculated using multivariable logistic regression to evaluate the association between the single lifestyle variables and the lifestyle score and the probability of detecting ACN.

RESULTS: In all 6315 women and men completed the lifestyle questionnaire, 3323 (53%) in the FIT arm and 2992 (47%) in the FS arm. This was 89% of those who participated in screening. ACN was diagnosed in 311 (5%) participants of which 25 (8%) were diagnosed with CRC. For individuals with a lifestyle score of two, three, four, and five-six, the ORs (95%CI) for the probability of ACN detection were 0.82 (0.45-1.16), 0.43 (0.28-0.73), 0.41 (0.23-0.64), and 0.41 (0.22-0.73), respectively compared to individuals with a lifestyle score of zero-one. Of the single lifestyle factors, adherence to non-smoking and moderate alcohol intake were associated with a decreased probability of ACN detection compared to being a smoker or having a high alcohol intake 0.53 (0.42-0.68) and 0.63 (0.43-0.93) respectively.

CONCLUSION: Adopted healthy behaviors were inversely associated with the probability of ACN detection. Lifestyle assessment might be useful for risk stratification in CRC screening.

Key words: Screening; Colorectal neoplasia; Lifestyle; Prevention; Health recommendations

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Core tip: Colorectal cancer (CRC) is a highly prevalent disease, developing from adenomas. In primary prevention of CRC, following public health recommendations such as non-smoking, normal body weight, physical activity, limited alcohol consumption and healthy diet is important. In the present study, we showed that adherence to multiple health recommendations was associated with decreased risk of detecting advanced colorectal neoplasia (ACN) in CRC screening. Regarding single health recommendations, non-smoking and moderate alcohol consumption were the most important lifestyle factors associated with low risk of ACN. Lifestyle assessment in CRC screening may therefore be used as a tool in risk stratification of participants.

Knudsen MD, de Lange T, Botteri E, Nguyen DH, Evensen H, Steen CB, Hoff G, Bernklev T, Hjartaker A, Berstad P. Favorable lifestyle before diagnosis associated with lower risk of screen-detected advanced colorectal neoplasia. *World J Gastroenterol* 2016; 22(27): 6276-6286 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6276.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6276>

INTRODUCTION

Worldwide and in Norway, colorectal cancer (CRC) is the second and third most common cancer type in women and men, respectively^[1]. In Norway, approximately 4000 new cases of CRC are diagnosed each year^[2]. CRC usually develops from an adenoma within 10-15 years on average, but very few adenomas (less than 10%) may progress to CRC within a lifetime^[3,4]. Still, early detection and removal of adenomas by screening may prevent CRC. Screening for CRC by either fecal immunochemical test (FIT), flexible sigmoidoscopy (FS) or guaiac-based fecal occult blood test (gFOBT) has been shown to reduce CRC mortality (FIT, FS and gFOBT) and CRC incidence (FS only)^[5-10]. Also, favorable lifestyle factors such as abstention from smoking, normal body mass, physical activity, limited alcohol consumption, and healthy dieting have been associated with a reduced risk of colorectal adenomas and CRC^[11,20]. Only a few studies have investigated the association between the number of healthy lifestyle factors and the risk of advanced colorectal neoplasia (ACN) in an average-risk population^[21,22]. When planning a national CRC screening program, it is important to identify and estimate the predictive value of lifestyle characteristics associated with detection of ACN. Stratifying of participants according to their risk profile will aid open possibilities for personalized CRC screening.

The aim of the present study was to investigate the association between the number of adherence of healthy lifestyle behaviors (based on Norwegian and international health recommendations) at the time of invitation to screening, and the probability of detecting ACN. Further, we aimed to investigate whether it was possible to predict screen-detected ACN based on single modifiable lifestyle factors. The present study is a lifestyle sub-study within the Bowel Cancer Screening in Norway (BCSN) - a randomized pilot study on a national program.

MATERIALS AND METHODS

Study population

The BCSN compares two screening modalities; five biennial rounds of FIT for occult blood in stools and a single FS^[23,24]. A total of 140000 women and men aged 50-74 at the time of randomization from two geographically defined areas in South-East Norway are randomly assigned (1:1 ratio) to one of the two

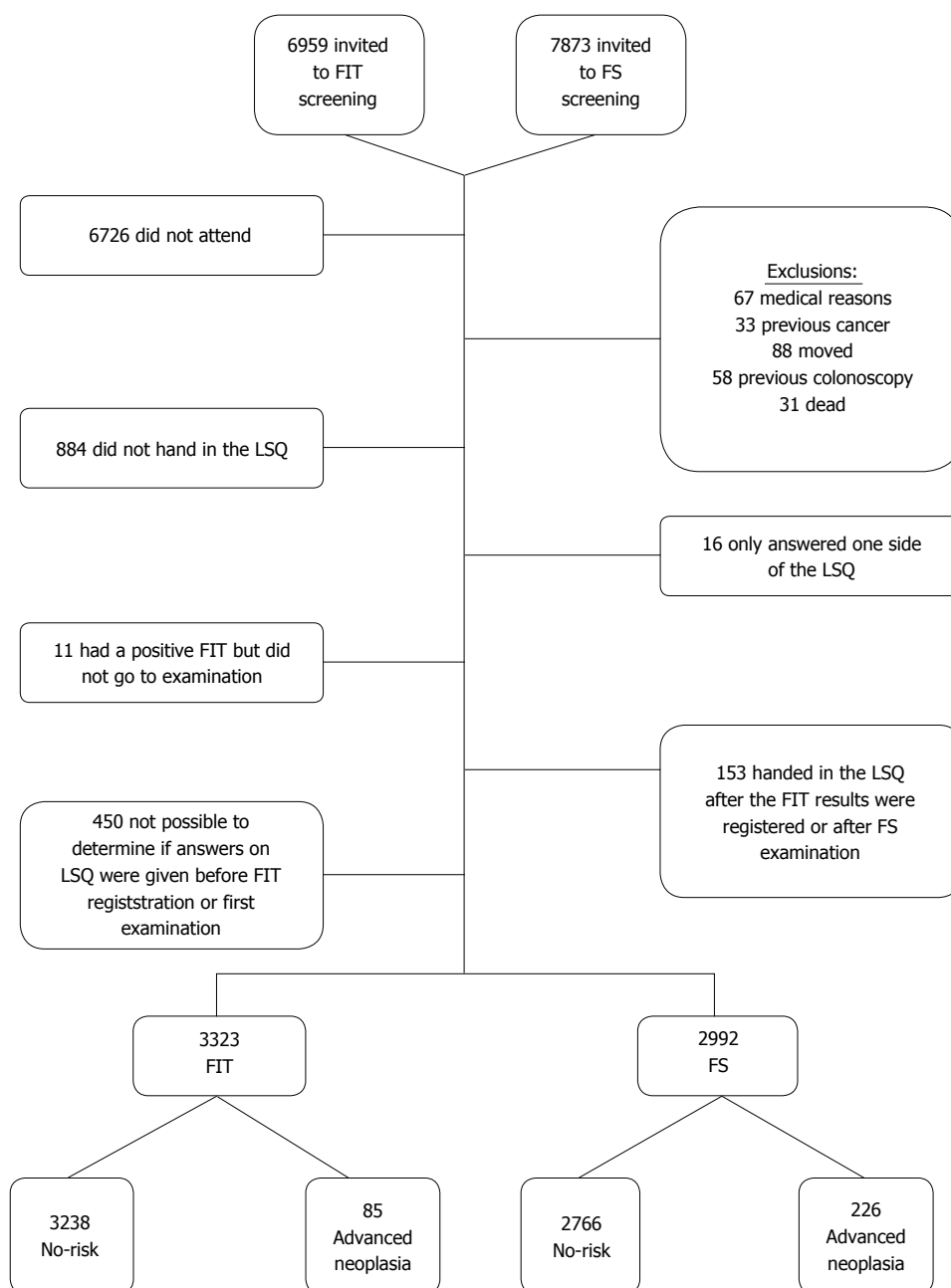


Figure 1 Flow chart of the participation in the screening and responders to the lifestyle questionnaire in the lifestyle sub-study of the bowel cancer screening in norway-a pilot study. FIT: Fecal Immunochemical Test; FS: Flexible Sigmoidoscopy; LSQ: Lifestyle questionnaire.

screening modalities. Invitations are scheduled from 2012 to 2018.

From November 2012 to September 2013, a total of 14832 individuals (6959 in the FIT arm and 7873 in the FS arm) were additionally invited to a separate lifestyle sub study (Figure 1). These were sent a two-page lifestyle questionnaire (LSQ) along with the invitation to the CRC screening. The LSQ was to be completed prior to the first CRC screening on paper or online prior to the availability of the screening results.

Examination procedures (outcome)

Examination procedures (outcome)
Participants assigned to FIT were mailed a self-administered kit with which they obtained a stool

sample and returned by mail to the laboratory. A test result of $\geq 75 \mu\text{g}$ hemoglobin/L buffer was considered a positive FIT. In the present study, FIT results were based on the first round out of five. FS was defined as positive if one of the following was detected or suspected: (1) any polyp ≥ 10 mm in diameter; (2) any adenoma with villous histology or high-grade dysplasia; (3) ≥ 3 adenomas; or (4) cancer. Participants with a positive screening result were referred to a follow-up colonoscopy. Based on the findings from the FIT, FS and follow-up colonoscopy, the participants were categorized into the following categories: (1) no adenomas (negative findings, non-neoplastic findings, other polyps); (2) low-risk

Table 1 Characteristics of participant's *n* (%)

Variable	Total (<i>n</i> = 6315) ¹ % column	Advanced colorectal neoplasia (<i>n</i> = 311) % row
Screening arm		
Fecal immunochemical test	3323 (53)	85 (3)
Flexible sigmoidoscopy	2992 (47)	226 (8)
Age		
mean (SD)	62.0 (7.0)	64.2 (6.9)
Sex		
Female	3255 (52)	118 (4)
Male	3060 (48)	193 (6)
Occupation		
Employed	3186 (50)	115 (4)
Unemployed	2944 (47)	189 (6)
Missing	185 (3)	7 (4)
Education length		
Primary school	1008 (16)	58 (6)
High school	2388 (38)	132 (5)
University/college studies of min. 2 yr	2636 (42)	104 (4)
Missing	283 (4)	17 (6)
Ethnicity		
Norwegian	5887 (93)	295 (5)
Not norwegian	361 (6)	12 (3)
Missing	67 (1)	4 (6)
Marital status		
Single, widowed, divorced	1195 (19)	62 (5)
Married, living together	5032 (80)	247 (5)
Missing	88 (1)	2 (2)
Chronic disease ²		
Yes	1593 (26)	86 (5)
No	4507 (74)	212 (5)
Missing	215 (3)	13 (6)
Whole meal bread slices per day		
< 1.5	1293 (20)	88 (7)
1.5	1895 (30)	83 (4)
3.5	2175 (34)	101 (5)
> 3.5	928 (15)	38 (4)
Missing	24 (0)	1 (4)

¹The number of replies may not sum to the total due to incomplete replies on the lifestyle questionnaire; ²Chronic disease was asked as: have you during the last 3 years had some chronic disease that limited your physical activity (e.g., problem with the hip or cardiovascular diseases) Demographic characteristics and whole meal bread consumption for participants in the lifestyle sub-study of the Bowel Cancer Screening in Norway - a pilot study.

adenomas (one or two adenomas with low-grade dysplasia of size < 10 mm in diameter); (3) high-risk adenomas (≥ 3 small adenomas or any adenoma of size ≥ 10 mm in diameter or adenomas with villous/tubulovillous/severe dysplasia); and (4) CRC [25]. ACN was defined as high-risk adenomas or CRC and served as the outcome. Individuals with low-risk adenomas or no adenomas were not considered to have ACN.

Exposure information - the LSQ

The LSQ consisted of questions copied from previous national surveys^[26,27] and previously used in the Norwegian Colorectal Cancer Prevention study (NORCCAP)^[28,29]. In the LSQ, participants were asked about their ethnicity, marital status and years of formal

education (Table 1). Participants were also asked to report smoking habits, weight, height, physical activity, and their consumption of alcohol and selected dietary items during the previous year. Reply options for smoking status were: "yes, daily", "yes, occasionally", "former", and "never smoked". Former smokers were further asked to state the number of years since smoking cessation. Three questions were asked regarding physical activity: "Have you during the last three years had any chronic disease that limits your physical activity?" (yes/no), and "How many times per week are you physically active for 30 min (1) with light or moderate intensity; and (2) with high intensity?". The reply options ranged from "never" to "more than seven times per week". Alcohol consumption was determined by two questions: (1) "How often have you consumed alcohol during the last year?", with reply options that ranged from "never" to "four-seven times per week"; and (2) "When consuming alcohol, how many glasses do you usually drink?". Consumption of fruit, berries and vegetables was determined by three separate questions regarding (1) fruit and berries; (2) raw vegetables; and (3) boiled vegetables. The consumption of red and processed meat was ascertained by three questions, where participants indicated their consumption at dinner of (1) steak, chops or similar; (2) hamburgers and dishes with minced meat; and (3) sausages. Consumption of fish was determined by one question on fatty fish. Bread consumption was ascertained by questions regarding the number of slices of bread with (1) a non-whole meal; (2) a partly whole meal; and (3) a whole meal. For the dietary items except bread, six frequency alternatives that ranged from "never/rarely" to "more than three servings per day" were given. For bread consumption, more than seven slices per day was the maximum.

Lifestyle variables

Individuals with certain smoking habits were divided into two groups: (1) smokers comprised current smokers, occasional smokers and those with smoking cessation ≤ 10 years ago; and (2) non-smokers who had never smoked or had stopped smoking > 10 years ago^[30]. Body mass index (BMI, kg/m²) was calculated from self-reported weight (kg) and height (cm). The two questions on physical activity (light/moderate and high intensity) were summed to the total number of times per week for 30 min or more. Alcohol consumption (glasses per week) was calculated by multiplying the answers to the two questions. The consumption of fruit and berries, raw vegetables and boiled vegetables was summed to a total fruit and vegetables consumed (F&V, servings per day). Consumption of total red and processed meat for dinner (R&P meat, servings per week) was calculated by summing the answers to the three questions on meat.

Table 2 Risk of advanced colorectal neoplasia by single lifestyle factors *n* (%)

Variable	Total (<i>n</i> = 6315) % column ¹	Advanced colorectal neoplasia (<i>n</i> = 311) % row	Lifestyle point ²	OR, (95%CI) ³
Smoking ⁴				
Smoker	1988 (31)	140 (7)	0	1.00 (Ref)
Non-smoker	4311 (68)	171 (4)	1	0.53 (0.42-0.68)
Missing	16 (0)	0 (0)		
Body Mass Index ⁵				
≥ 25.0	3658 (59)	205 (6)	0	1.00 (Ref)
< 25.0	2542 (41)	97 (4)	1	0.78 (0.60-1.01)
Missing	115 (2)	9 (8)		
Physical activity 30 min, times per week				
< 7	4480 (71)	238 (5)	0	1.00 (Ref)
≥ 7	1504 (24)	58 (4)	1	0.81 (0.60-1.09)
Missing	331 (5)	15 (5)		
Alcohol, glasses per week				
≤ 14 for male, ≤ 7 for female)	5525 (88)	264 (5)	1	0.63 (0.43-0.93)
Above	514 (8)	34 (7)	0	1.00 (Ref)
Missing	276 (4)	13 (5)		
Fruits and vegetables, servings per day				
< 3	4621 (73)	244 (5)	0	1.00 (Ref)
≥ 3	1446 (23)	52 (4)	0.5	0.93 (0.67-1.27)
Missing	248 (4)	15 (6)		
Red and processed meat, servings per week				
> 4	2059 (32)	108 (5)	0	1.00 (Ref)
≤ 4	4093 (65)	196 (5)	1	0.98 (0.76-1.26)
Missing	163 (3)	7 (4)		
Fatty fish, servings per week				
< 1	1257 (20)	78 (6)	0	1.00 (Ref)
≥ 1	5023 (79)	232 (5)	0.5	0.79 (0.60-1.05)
Missing	35 (1)	1 (3)		
C-statistics				0.72 (0.69-0.75)

¹The number of replies may not sum to the total due to incomplete replies on the lifestyle questionnaire; ²One point for each lifestyle adherence, except only 1 point if adherence to both: fatty fish and fruits and vegetables; ³Models are adjusted for: age (continuously), screening arm (flexible sigmoidoscopy, fecal immunochemical), gender (women, men), center (Moss, Bærum), education (primary school, high school, university/college studies of minimum 2 yr) and whole meal bread. Lifestyle factors (Body Mass Index, smoking, alcohol, physical activity, fruits and vegetables, red and processed meat and fatty fish) were mutually adjusted for each other; ⁴18% current smoker, 14% ≤ 10 yr stopped, 28% > 10 yr stopped, 40% never smokers; ⁵43% had a Body Mass Index of 25.0-30.0; 16% had a Body Mass Index of > 30.0. Lifestyle characteristics, point for the lifestyle score, odds ratio (OR) and 95%CI between the single lifestyle factors and the risk of advanced colorectal neoplasia by multivariable logistic regression for participants in the lifestyle sub-study of the Bowel Cancer Screening in Norway - a pilot study.

Categorization of variables

A lifestyle score was generated to reflect the number of favorable lifestyle factors fulfilled. The score was based on the following factors: smoking habits, BMI, physical activity, and consumption of alcohol, F&V, R&P meat, and fatty fish. These factors were chosen for their modifiable character and for being central in Norwegian and international health recommendations. Each of the single lifestyle factors was dichotomized to reflect adherence to health recommendations^[31-33]. Each participant was assigned one point for each fulfilled lifestyle criterion, except F&V and fish for which both had to be fulfilled to earn one point (Table 2). The total number of points in the lifestyle score ranged from zero (poorest) to six (best) (Table 3).

The Norwegian health recommendation for F&V is a minimum of five servings per day^[31]. However, only 4.5% of the participants in the present study reported this value. According to a national survey, 25% of the Norwegian population fulfill the recommendation for

F&V^[34]. As 25% of the participants in our study had a total consumption of three or more servings per day, we used this cut off value to mark favorable F&V consumption in our analyses.

Statistical analysis

The odds ratio (OR) and 95%CI were calculated using multivariable logistic regression models to evaluate the association between single lifestyle factors and the lifestyle score with the risk of ACN. Single lifestyle factors were analyzed as dichotomous variables using values that did not reach the lifestyle score point as the reference category. To gain enough participants for the reference group, the scores of zero or one were pooled (zero-one) in the lifestyle score analyses. Statistical models that examined the association between single lifestyle factors and the risk of ACN were mutually adjusted for the remaining single lifestyle factors. Moreover, all multivariable models were adjusted for gender, age at invitation (continuous), years of formal

Table 3 Risk of advanced colorectal neoplasia by the lifestyle score *n* (%)

	Total (<i>n</i> = 6315) % column ¹	Advanced colorectal neoplasia (<i>n</i> = 311), <i>n</i>	Absolute risk estimates per 1000 ⁴	OR, (95%CI) ²	<i>P</i> trend ⁵
Lifestyle score ³					< 0.001
0-1	371 (6)	32	86.3	1.00 (Ref)	
2	1248 (20)	92	73.7	0.82 (0.45-1.16)	
3	1749 (27)	71	40.6	0.43 (0.28-0.73)	
4	1312 (21)	46	35.1	0.41 (0.23-0.64)	
5-6	684 (11)	21	30.7	0.41 (0.22-0.73)	
Missing	951 (15)	49			
C-statistics				0.71 (0.68-0.74)	

¹The number of replies may not sum to the total due to incomplete replies on the lifestyle questionnaire; ²The model was adjusted for age (continuously), screenings arm (fecal immunochemical test, flexible sigmoidoscopy), gender (women, men), center (Moss, Bærum), education (primary school, high school, university/college studies of minimum 2 years) and whole meal bread; ³One point for each lifestyle adherence, except only 1 point if adherence to both: fatty fish and fruits and vegetables; ⁴Absolute risk calculated: with in each score: (*n* ACN/Total *n*)*1000; ⁵*P* trend was calculated using the lifestyle score as continuous. Lifestyle score characteristics, odds ratio (OR) and 95%CI for the risk of advanced colorectal neoplasia by multivariable logistic regression for participants in the lifestyle sub-study of the Bowel Cancer Screening in Norway - a pilot study.

education (primary school, high school, university/college studies of minimum 2 years), screening modality (FIT or FS), screening center (Moss or Bærum Hospital) and whole meal bread consumption (< 1.5, 1.5, 3.5, or > 3.5 slices per day). These factors were selected based on *a priori knowledge* of the association of these factors with CRC^[12,14,35] and colorectal adenomas^[15-19,36]. In spite of the convincing evidence of an inverse association with the risk of CRC, we included whole meal bread consumption as a covariate and not in the lifestyle score. This was based on the uncertainty of bread consumption alone to mark dietary fiber/wholegrain intake.

Omitted values for the lifestyle factors in question were treated as fixed/dummy values. If a participant had a missing value in a lifestyle factor used to create the lifestyle score, this factor was considered missing in the lifestyle score for that participant.

Multivariable restricted cubic spline logistic regression models with three knots were used to analyze the functional form, and *P* trend, of the relationship between single lifestyle factors as continuous (BMI, physical activity, alcohol consumption, F&V and R&P meat) and the probability of ACN^[37].

C-statistics were calculated to examine how well the multivariable logistic regression models discriminated between participants who were and were not diagnosed with ACN, estimating C. C is an estimate for the sensitivity and specificity of the statistical model^[38].

In the sensitivity analysis, we tested the effect of the lifestyle score on the risk of ACN by including fewer than six lifestyle factors. A change in the order of the

included factors was also tested. We then stratified the analyses for gender, smoking and screening modality. Likelihood ratio tests were also performed to test for effect modification.

Absolute risk was calculated for each lifestyle score category as well.

Analyses were performed using STATA™ software, version 13.1 (Stata Corp, College Station, Texas, United States) and R software (The R Foundation for Statistical Computing Platform 2014, Free Software Foundation, Boston, MA, United States). The statistical methods of this study were reviewed by statistician Edoardo Botteri from Department bowel cancer screening, Cancer Registry of Norway.

Ethics

The Regional Research Ethics Committee of South-East Norway and the Norwegian Data Inspectorate approved the study protocol (approval No. 2011/1272). The participants gave their consent to participate in the lifestyle study by completing and returning the mailed questionnaire.

RESULTS

In all, 6315 participants were included in the present study; 53% (*n* = 3323) were in the FIT arm and 47% (*n* = 2992) were in the FS arm (Figure 1). The participation rate for both the screening examination and successful completion of the LSQ was 43% (48% in the FIT arm and 38% in the FS arm). ACN was diagnosed in 311 (4.9%) participants of which 25 (8%) were diagnosed with CRC.

A higher proportion of participants who were diagnosed with ACN were unemployed, smokers, and had a BMI ≥ 25 kg/m² compared with participants without ACN (Tables 1 and 2).

Age at invitation, male gender and randomization to FS screening compared with FIT screening were associated with an increased risk of ACN (results not shown). The adjusted OR for the risk of ACN was 0.53 (95%CI: 0.42-0.68) for non-smokers compared with smokers. The adjusted OR for the risk of ACN was 0.63 (95%CI: 0.43-0.93) in individuals with moderate alcohol consumption compared with those with a consumption above the moderate level. No significant associations were observed between BMI, the single dietary factors or physical activity and the risk of ACN (Table 2).

Figure 2 shows the functional form of the relationship between single continuous lifestyle factors (except smoking and consumption of fatty fish) and the probability of ACN. Based on the multivariable restricted cubic spline logistic regression, continuous BMI (*p*-trend) was associated with an increased probability of ACN (Figure 2).

The lifestyle score was inversely associated with the risk of ACN, *P* trend < 0.001. The absolute number of

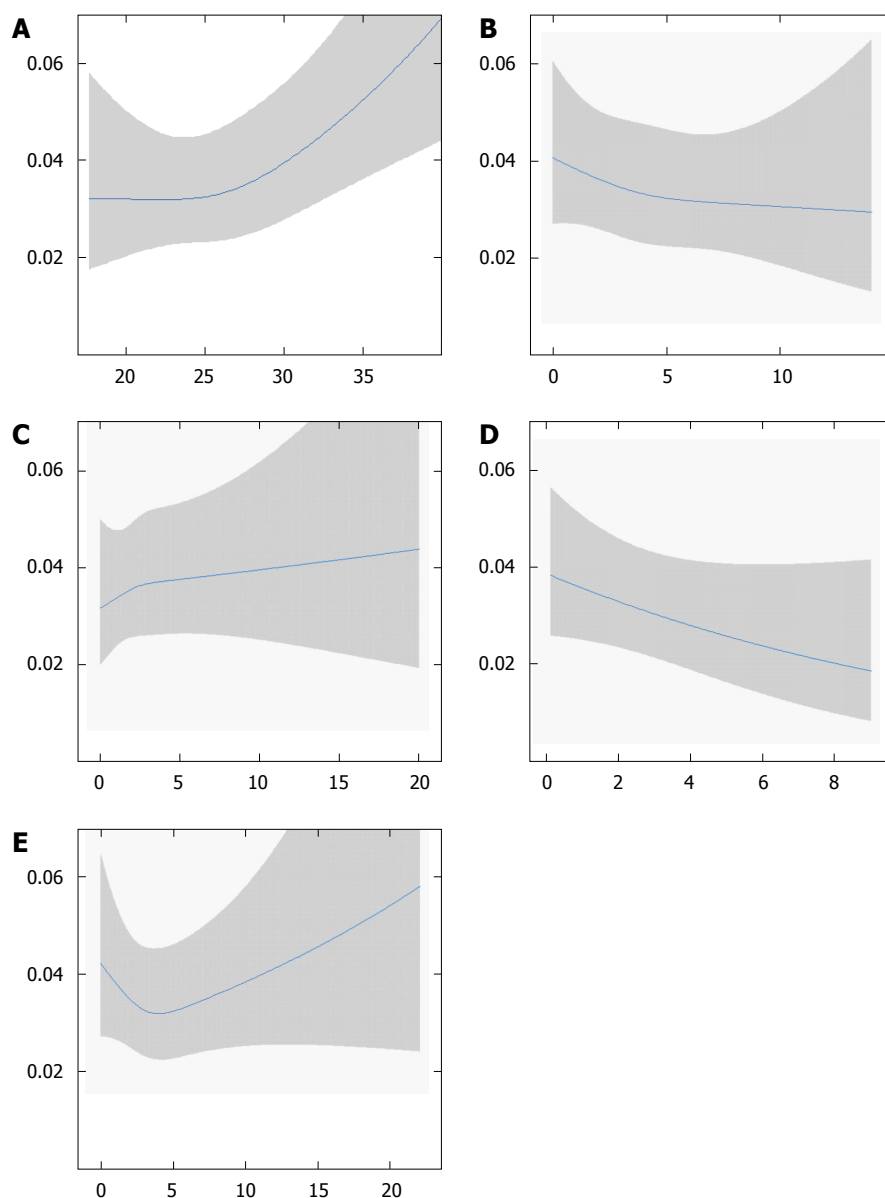


Figure 2 Relationship between advanced colorectal neoplasia and Body Mass Index^a (A) physical activity(B) alcohol consumption(C) fruit and vegetable consumption (D); and (E) red and processed meat consumption. The relationship is modeled by cubic splines logistic (continuous line) with 3 knots. The model is based on 95%CI, which are reported by the grey areas. All models are adjusted for: age (continuously), screening arm (flexible sigmoidoscopy, fecal immunochemical), gender (women, men), center (Moss, Bærum), education (primary school, high school, university/college studies of minimum 2 year) whole meal bread, fatty fish and smoking (smoker, non-smoker). The lifestyle factors (BMI, physical activity, alcohol consumption, fruit and vegetables and red and processed meat) were mutually adjusted for each other. A: BMI 16.5-40 (> 40 does not appear in the figure); B: Physical activity 0-14 times for 30 min per week; C: Alcohol consumption 0-30 glasses per week; D: Total fruit and vegetable consumption 0-9 servings per day; E: Red and processed meat consumption 0-15 servings per week. ^a $P < 0.001$, increase/decrease in the regression coefficient significantly different from 0.

individuals with ACN decreased from 86.3 to 30.7 per 1000 by increase of the lifestyle score from zero-one to four or five-six. The adjusted OR for ACN was 0.41 (95%CI: 0.22-0.73) for participants with a lifestyle score of five-six compared with those with a score of zero-one (Table 3).

Sensitivity analyses, which included fewer than six lifestyle factors and tested the order of the lifestyle factors that were included in the score showed that non-smoking was the most important factor in the reduction of the OR for ACN. Lowest risk for ACN was associated with adherences to the four lifestyle factors;

non-smoking, normal BMI, adequate physical activity and moderate alcohol consumption, compared to adherence to none or only one of these behaviors (OR = 0.31, 95%CI: 0.17-0.59). The addition of points for a healthy diet did not reduce the risk of ACN further. Results did not change when stratifying for gender (results not shown). When stratifying for smoking status, the risk of ACN was reduced for non-smokers having a moderate alcohol consumption, compared to non-smokers with a consumption above moderate 0.76 (95%CI: 0.33-0.99). For smokers the risk of ACN was reduced for those with a high F&V intake 0.45 (95%CI:

0.23-0.88) compared to those with a low F&V intake. Results for the remaining variables were not changed when stratifying for smoking status. When the results were stratified according to screening modality, we observed a significant inverse association between the lifestyle score and ACN only in the FS arm. Results from the FIT arm showed a similar trend but did not reach statistical significance. There was no indication of effect modification by the screening arm in the association between the lifestyle score or the single lifestyle factors, and ACN risk (*P* values for interaction ranged from 0.178 to 0.984).

The results from the C-statistics indicate that the models used were acceptable in the discrimination between participants with and without ACN, as the C values were between 0.70-0.80^[39]: C-statistics = 0.72 (95%CI: 0.69-0.75) for ACN and single lifestyle factors, C-statistics = 0.71 (95%CI: 0.68-0.74) for ACN and the lifestyle score (Table 2 and 3).

DISCUSSION

In the present study, we observed a low risk of ACN in CRC screening participants who fulfilled several healthy lifestyle factors reflecting adherence to public health recommendations. The present study suggests that favorable health behavior, particularly non-smoking and moderate alcohol consumption predicts a low risk of CRC in a general population. These results highlight the potential of lifestyle assessment as a tool in risk stratification of participants in CRC screening.

Studies that investigate the association between lifestyle factors and the risk of ACN in a CRC screening setting are unusual. In the FS-based NORCCAP study, similar associations were observed between smoking, physical activity, and consumption of F&V, R&P meat and fatty fish and the risk of ACN^[29], but no information on alcohol was collected. A Chinese CRC screening study^[40] and an American case-control study^[21] investigated the association between ACN and a lifestyle score. Our results are in agreement with results from these two studies, although some differences were seen in the factors that were considered for the lifestyle scores. The Chinese study included both non-modifiable factors (e.g., family history of CRC, diabetes, age and gender) along with the modifiable risk factors smoking and BMI in their scoring system^[40]. The American study included calcium intake and the use of non-steroidal anti-inflammatory drugs in the lifestyle score^[21].

Three European studies and one American cross-sectional study on colonoscopy screening concluded that through a set of participant characteristics, it might be possible to identify a high-risk population for adenomas and to target that population for CRC-screening^[41-44]. These studies included both modifiable and non-modifiable risk factors such as family history of CRC, previous detection of polyps, diabetes and FIT results. Based on the C-statistics that were obtained

in these previous European^[41-43], Chinese^[40] and American^[44,45] studies, our model that included only modifiable risk factors for CRC was equally able to discriminate between the participants diagnosed and not diagnosed with ACN. This suggests that modifiable lifestyle assessment in CRC screening could be used as a tool in risk stratification of participants and to identify high-risk participants in CRC-screening. Two large prospective cohort studies, one in the European Prospective Investigation into Cancer and Nutrition and a Danish study, showed that lifestyle scores based on modifiable factors similar to those in the present study predict the risk of future CRC^[11,13]. The present study with the cross-sectional design is not comparable with those studies. However, we observed similar associations between current lifestyle and ACN to those in the large cohort studies, although current lifestyle at the time of screening might not represent the lifestyle at the time of onset of adenoma development.

The present study has several strengths. The population registry based randomization limits the risk of selection bias. Compared to other European population-based trials in CRC screening^[41,46], compliance to complete the LSQ among screening participants (89%) was high. This may have reduced the risk of selection bias, suggesting that the results are representative for CRC screening participants. Information bias was unlikely due to the study design that entailed the completion of the LSQ before the screening results were available. The limitations of the present study include that we had no information on potential confounders such as energy intake, use of non-steroidal anti-inflammatory drugs and hormone therapy in women, or family history of CRC^[14,47,48]. However, because BMI and physical activity were included in the statistical models, the risk of confounding by energy intake should be limited.

The specific questions in the LSQ have been used in other validated questionnaires^[26,27], however, the brevity of the LSQ, which was designed to require less than 10 min for completion, may have sacrificed details on dietary consumption. The overall participation rate of 43% might question the present study's representativeness of the general Norwegian population. Prevalence of smoking (18%, including occasional smokers) in the present study compared to the 15%-20% daily smokers in the Norwegian population of similar age^[49] suggests representative lifestyle characteristics in the study population. We acknowledge that FIT and FS as screening modalities might have caused some false negative screening results (misclassification) because one round of FIT has limited sensitivity to discover ACN, and FS only involves the distal segments of the colon^[50,51]. Any misclassification might have attenuated the association between the lifestyle factors and the risk of ACN. This added to the brevity of the LSQ might also be a reason why no significant linear association was observed

for the single lifestyle factors. Furthermore, the lack of significant associations between the single lifestyle factors, the lifestyle score, and ACN in the stratified FIT arm might be explained by misclassification because it was based on only the first of five FIT rounds.

Alternative lifestyle scores based on quartiles of the single lifestyle factors could have been considered. However, quartiles are not as easy interpretable for the general population as the present lifestyle score which reflects adherence to public health guidelines.

In summary, the simplicity of the lifestyle score, which was created to reflect healthy lifestyle behaviors in the present study, makes it an easy tool to implement in CRC screening. This score provides straightforward information on the probability of detecting ACN in a general population. Furthermore, the lifestyle score could be considered used as a risk stratification tool when predicting the risk of detecting ACN in CRC screening. We showed that the probability of detecting ACN was 59% lower in screening participants having a score of minimum four compared to a score of zero-one. Based on this population-based screening study, the single lifestyle factor with the lowest probability of detecting ACN was non-smoking. However, moderate alcohol consumption was also effective for non-smokers and F&V for smokers. When the results from the present study are generalized, one should consider that the study was based only on Norwegians.

In conclusion, the present study suggests that the probability of detecting ACN in CRC screening is low in participants who adhere to multiple favorable lifestyle behaviors. Lifestyle assessment may be useful for risk stratification in CRC screening.

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COMMENTS

Background

Single lifestyle factors are associated with the risk of colorectal adenomas and cancer. In the planning of a colorectal cancer (CRC) screening programs, it is important to estimate the predictive value of lifestyle factors for detection of advanced colorectal neoplasia (ACN).

Research frontiers

The aim of this study was to investigate whether detection of ACN was associated with the number of modifiable healthy lifestyle factors in CRC screening participants.

Innovations and breakthroughs

The present study shows that the number of adopted healthy lifestyle behaviors reflecting public health recommendations predicts the outcome in a CRC screening examination. Screening participants adhering to at least four healthy behaviors have 59% lower risk of ACN detected at screening, when compared to participants adhering to none or only one healthy behavior.

Applications

Adhering to several health recommendations is effective in the prevention of

CRC. Assessment of lifestyle factors at CRC screening may be useful as a tool in risk stratification of participants.

Peer-review

CRC is one of the most common cancer types in both women and men. This is an important study, investigating whether detection of ACN was associated with the number of modifiable healthy lifestyle factors in CRC screening participants. The study shows a lower risk of ACN with increased number of healthy lifestyle factors. The results were interesting and the article is of great interest for the readers of the journal.

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Prospective Study

Total and not bevacizumab-bound vascular endothelial growth factor as potential predictive factors to bevacizumab-based chemotherapy in colorectal cancer

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Abstract

AIM: To identify suitable biomarkers of response to bevacizumab (BV) - it remains an open question. The measurement of serum vascular endothelial growth factor (VEGF) has been proposed as a predictive factor for this drug, even if literature data are contradictory.

METHODS: We prospectively evaluated the role of BV, total and not BV-bound VEGF and angiopoietin-2 (Ang-2) serum levels as potential predictive factors of response for BV in combination with an oxaliplatin-based chemotherapy. BV, Ang-2, total and not BV-bound VEGF levels were measured at baseline, before

2nd and 5th cycle of oxaliplatin-based chemotherapy in 20 consecutive metastatic colorectal cancer patients.

RESULTS: Results were correlated to response to treatment. Variability in BV levels have been found, with decreased level in less responding patients. In particular, the concentration of BV increased of 3.96 ± 0.69 folds in serum of responsive patients after 3 more cycles of therapy compared to those with stable or progressive disease with a 0.72 ± 0.25 and 2.10 ± 0.13 fold increase, respectively. The determination of free and total VEGF demonstrated that the ratio between the two values, evaluated immediately before the 2nd and the 5th cycle of therapy, decreased from $26.65\% \pm 1.33\%$ to $15.50\% \pm 3.47\%$ in responsive patients and from $53.41\% \pm 4.75$ to $34.95\% \pm 2.88\%$ in those with stable disease. Conversely, in those with progression of disease, the ratio showed the opposite behavior coming up from $25.99\% \pm 5.23\%$ to $51.71\% \pm 5.28\%$. The Ang-2 levels did not show any relationship.

CONCLUSION: Our data show that the ratio of not BV-bound VEGF to total VEGF serum and BV plasma concentrations for predicting the response to BV plus oxaliplatin-based chemotherapy could be a promising biomarker of response to BV.

Key words: Bevacizumab; Vascular endothelial growth factor; Angiopoietin 2; Metastatic colorectal cancer; Biomarker; Predictive factor

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Core tip: In the main topic of the identification of possible reliable markers to predict response to anti-angiogenic therapy, our paper represents an original contribution describing the role of not bevacizumab (BV)-bound vascular endothelial growth factor (VEGF)/total VEGF ratio and of bevacizumab serum level as predictors of response in a consecutive series of patients with metastatic colorectal cancer. In this paper the rediscovery of the predictive role of traditional biomarkers as not BV-bound VEGF/total VEGF plasma ratio together with the results of the bevacizumab pharmacokinetic in response, stable disease and progression settings of patients with metastatic colorectal cancer treated with bevacizumab plus oxaliplatin based chemotherapy supported our hypothesis.

Azzariti A, Porcelli L, Brunetti O, Del Re M, Longo V, Nardulli P, Signorile M, Xu JM, Calabrese A, Quatralo AE, Maiello E, Lorusso V, Silvestris N. Total and not bevacizumab-bound vascular endothelial growth factor as potential predictive factors to bevacizumab-based chemotherapy in colorectal cancer. *World J Gastroenterol* 2016; 22(27): 6287-6295 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6287.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6287>

INTRODUCTION

Colorectal carcinoma (CRC) is the second leading cause of death from cancer in Europe and North America, with approximately one million new cases and half a million deaths per year worldwide^[1]. In recent years treatment of metastatic disease has undergone a major evolution with the introduction of biologic drugs alone or in combination with chemotherapy regimen^[2]. In particular, the addition of bevacizumab (BV), a humanized recombinant monoclonal antibody anti-vascular endothelial growth factor-A (VEGF-A), to standard chemotherapy regimens resulted associated to survival with an improvement in overall survival (OS) with respect to chemotherapy alone in patients with metastatic disease^[3,4]. Nevertheless, differently from the anti-epidermal growth factor receptor monoclonal antibodies, for which the RAS state is a validated predictive biomarker^[5,6], to date, there is no evidence of predictive markers of response to BV. Identification of predictive biomarkers would allow selection of patients most likely to benefit from treatment with BV, thereby avoiding toxicities. Several potential biomarkers have been studied and proposed as predictors of response to BV^[7,8], among them the VEGF. VEGF is overexpressed in 40%-60% of CRC and is correlated with intratumoral vascular density^[9]. Indeed, a meta-analysis published by Des Guetz *et al*^[10] reported the prognostic biomarker value of VEGF: the analysis of published studies relating intratumoural microvessel density (MVD) or VEGF expression, highlighted that high MVD and VEGF expression significantly predict poor progression free survival (PFS) and OS. However, pre-treatment tumor VEGF expression or circulating VEGF levels are not predictive of response to BV, but data obtained from a study that retrospectively analysed samples from two randomized phase III studies (HORIZON II and III), evaluating the prognostic and/or predictive value of VEGF signaling showed that high baseline VEGF levels were associated with worse outcomes for PFS and OS independent of treatment^[11]. Anyway, these experimental findings that did not find any association between VEGF and response to treatment, could be related to a lack of differentiation between total VEGF (including BV-bound VEGF and not BV-bound VEGF) and not BV-bound VEGF. According to these data, a decrease of not BV-bound VEGF levels during treatment with BV has been observed using immunodepleted plasma samples^[12]. On this regard, Loupakis *et al*^[13] analysed plasma not BV-bound VEGF in metastatic CRC (mCRC) patients during BV treatment, after an immunodepletion procedure able to eliminate, among the other immunoglobulins, BV and BV-bound VEGF, suggesting that the anti-VEGF antibody significantly reduces not BV-bound and biologically active VEGF concentrations. Angiopoietin-2 (Ang-2), an inhibitory ligand of the Tie-2 receptor, stored in the Weibel-Palade bodies

of endothelial cells^[14], has been described as an opponent of vascular normalization that prevents blood vessels from becoming structurally and functionally stabilized^[15,16]. High levels of Ang-2 contrasting the normalization of tumor vessels mediated by BV may reduce the delivery of chemotherapeutic drugs into tumor tissues^[17,18]. Moreover, BV has a high inter-individual variability in terms of pharmacokinetic, with a half-life ranging between 11 and 50 d. Blood concentrations of BV above and below the median have been reported to correlate with its toxicity and efficacy profile, respectively^[19].

In the present study, we prospectively measured not BV-bound VEGF, Ang-2 and plasma BV levels of mCRC patients treated with BV in combination with oxaliplatin-based chemotherapy regimen, in order to find predictive biomarkers of response to BV-therapy.

MATERIALS AND METHODS

Patients

Consecutive patients aged ≥ 18 years, with histologically confirmed mCRC, an Eastern Cooperative Oncology Group performance status (ECOG PS) ≤ 2 , measurable disease, adequate hepatic and renal function, and no contraindications to BV therapy were included from December 2012 until January 2014. These patients received BV (5 mg/kg) and chemotherapy, consisting of FOLFOX-4 (leucovorin 200 mg/m² per day as a 2-h infusion followed by bolus 5-fluorouracil 400 mg/m² per day and a 22-h infusion of 5-fluorouracil 600 mg/m² per day repeated for 2 consecutive days every 2 wk, with the addition of oxaliplatin 85 mg/m² on day 1) or XELOX-2 (oxaliplatin 100 mg/m² per day followed by oral capecitabine 1000 mg/m² twice daily on days 1 through 7 of a 14-d cycle) as first or second line of treatment^[20,21]. Response to treatment was evaluated between the 4th and the 5th cycle according to the Response Evaluation Criteria in Solid Tumors (RECIST) definition 1.1^[22]. To exclude that the associated chemotherapeutic regimen could affect VEGF and Ang-2 levels, four patients in adjuvant treatment were included as a control arm. The study was approved by the Ethics Committee of the National Cancer Research Centre "Istituto Tumori Giovanni Paolo II" of Bari and informed consent was obtained from all patients enrolled in the study.

Blood samples collection and biomarkers detection

Venous blood was drawn at day 1 (baseline), and immediately before the 2nd and 5th cycle of chemotherapy. For control arm, venous blood was drawn only at the baseline and immediately before the 2nd cycle of therapy.

Plasma preparation: Whole blood was collected into commercially available EDTA-treated tubes and cells removed from plasma by centrifugation for 15 min at

2000 $\times g$ at 4 °C, depleting also platelets. The plasma fractions were divided in aliquots, frozen and stored at -80 °C until assayed.

Serum preparation: After allowing the blood to clot by leaving it undisturbed at room temperature for 30 min, the clot was removed by centrifuging at 2000 $\times g$ for 15 min at 4 °C. The serum fractions were divided in aliquots, frozen and stored at -80 °C until assayed.

Plasma VEGF, not BV-bound VEGF and Ang-2

detection: VEGF and Ang-2 plasma levels were measured by means of the ELISA Quantikine DVE00 and DANG 20 Kits (R&D Systems, Minneapolis, MN, United States), respectively. The optical density was determined using the multilabel plate reader Victor 3 (Perkin Elmer) set to 450 nm, with a wavelength correction set to 540 nm. To measure not BV-bound VEGF concentrations, plasma samples were immunodepleted as described by Loupakis *et al.*^[13]. Briefly, plasma samples were immunodepleted using Protein G-Sepharose 4 Fast Flow beads (Pharmacia Biotech, Uppsala, Sweden). Preliminarily, the beads were washed twice in PBS, then, these was reconstituted to 50% (v/v) protein G-sepharose in PBS. To deplete plasma samples of BV plus BV-bound VEGF, 100 μ L of protein G slurry was added to 200 μ L of plasma samples and incubated at 4 °C for 4 h. After centrifugation (2 min at 10000 rpm), 200 μ L of plasma supernatants was removed and the immunodepletion was repeated by the addition of 100 μ L of protein G slurry and overnight incubation at 4 °C. Each plasma sample was then assayed for human VEGF concentrations using the ELISA kit.

BV detection: The serum concentration of BV was measured with a home-made enzyme-linked immunosorbent assay (ELISA) kit^[23]. Briefly, microwell plates (Immuno 96 Micro Cell solid plates; Nunc, Roskilde, Denmark) were coated with 100 μ L/well recombinant human 1.0 μ g/mL VEGF165 (R&D Systems, Minneapolis, MN) at a concentration of 1.0 μ g/mL overnight at 4 °C. After three wash steps with PBS plus 0.05% Tween-20, the blocking of the wells was done with 3% BSA/PBS overnight at 4 °C (200 μ L/well) to reduce non-specific binding. After five wash steps with PBS plus 0.05% Tween-20, 50 μ L/well of each serum sample (diluted 1:1000 in PBS) and 50 μ L/well of different concentrations of the standard were added to the plates. Incubation was overnight at 4 °C. A standard curve was prepared with BV ranging from 1 ng/mL to 5000 ng/mL. The bound BV was made detected with 1 μ g/mL of horseradish peroxidase-goat anti-human IgG (H + L) conjugate (Invitrogen Corporation, Carlsbad, CA) after an incubation of wells for 3 h at room temperature. After five wash steps with PBS plus 0.05% Tween-20, the substrate used

Table 1 Clinical characteristics of patients

	Number of patients (n = 20)
Sex	
Male	11/20
Female	9/20
Age	
mean	64.2
range	28-80
Number of metastatic sites	
1-2	7/20
≥ 3	13/20
Tumor differentiation	
G1-G2	12/20
G3	8/20
Associated chemotherapy	
Folfox4	6/20
Bi-weekly XELOX	14/20
Line of therapy	
First	17/20
Second	3/20
Response to treatment	
PR	6/20
SD	9/20
PD	5/20
Median number of cycles	9 (range: 5-14)

PR: Partial response; SD: Stable disease; PD: Progression disease.

was BM Blue POD substrate (Roche, United States) stopped with 1 mol/L HCl (100 μ L). Absorbance was read at 450 nm on a multilabel plate reader Victor 3 (Perkin Elmer). In the plot, the BV serum accumulation is expressed as a ratio between drug concentration before the 5th cycle and before the 2nd cycle.

Statistical analysis

All samples determinations were performed in triplicate, and results have been expressed as the mean \pm SD, unless otherwise indicated. Statistical differences *in vitro* data were assessed by the Student-Newman-Keuls test. *P* values lower than 0.05 were considered significant.

RESULTS

Twenty mCRC patients were evaluated for the changes of BV, not BV-bound VEGF, total VEGF and Ang-2 plasma concentrations in function of time of BV plus chemotherapy administration. Eleven patients were male and 9 were female with a median age of 64.2 years (range: 28-75 years). Thirteen patients had 3 or more metastatic sites and the majority received a first line therapy. Five of them had progression disease (PD), while 9 and 6 had stable disease (SD) and partial response (PR), respectively at first evaluation (Table 1).

Assessment of BV serum levels showed that immediately before the 2nd cycle the level was quite similar between all patients, measuring 2.61 ± 1.10 , 5.51 ± 5.28 and 2.33 ± 0.94 μ g/mL in PR, SD and PD, respectively (Table 2). Conversely, the modification of the drug serum concentration before the 5th cycle,

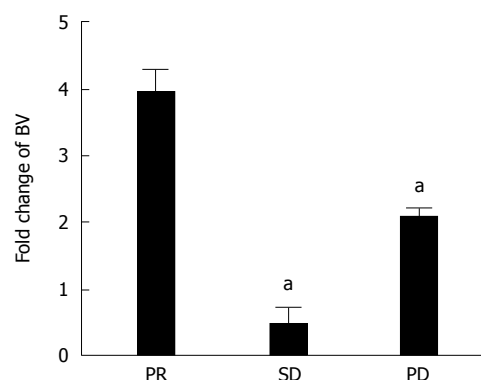


Figure 1 Increase of bevacizumab level in serum. Increase of the serum concentration of the drug in function of time; the serum was withdrawn before the 2nd and the 5th cycle of therapy. Results are divided in three histograms, representative of the fold change of bevacizumab (BV) in patients who showed partial response (PR), stable disease (SD) and progression disease (PD). ^a*P* < 0.05, BV of patients with SD or PD vs with PR.

reported as the ratio between BV total amount, determined before the 5th and before the 2nd cycle, was different among patients with PR compared to the others (Figure 1 and Table 2). In particular, the first showed a 3.96 ± 0.69 -fold increase in BV serum level after three more cycles of therapy compared to those with SD and PD with a 0.72 ± 0.25 and 2.10 ± 0.13 -fold increase, respectively.

A preliminary control step was the measurement of VEGF and Ang-2 levels in both immune depleted and not immunodepleted plasma from 4 CRC patients treated with adjuvant FOLFOX-4 regimen. These results allowed us to exclude that the associated chemotherapeutic regimens could affect VEGF and Ang-2 levels. Similar results, obtained in all pairs of samples, demonstrated that neither the immunodepletion procedure nor the chemotherapy affected the measurement of VEGF and of Ang-2 (Table 3).

VEGF basal levels have been determined into plasma of all the patients. VEGF basal levels were 376.39 ± 221.97 pg/mL, 308.80 ± 177.76 pg/mL and 395.98 ± 283.00 pg/mL in PR, SD and PD respectively. The determination of VEGF as a total protein showed that its plasma levels are different in the population (Table 2) without a correlation with the response to therapy, in agreement with data reported in literature^[24].

Moreover, assessment of VEGF as total and not BV-bound was carried out in plasma samples of each patient before the 2nd cycle of therapy and before the 5th cycle. Ratio of median value of VEGF not BV-bound /VEGF total was calculated for all patients. In particular, VEGF not BV-bound /VEGF total ratios determined before the 2nd cycle were 26.65 ± 1.33 , 53.41 ± 4.75 and 25.99 ± 5.23 for PR, SD and PD, respectively. Interestingly, the VEGF not BV-bound /VEGF total ratios before the 5th cycle were 15.50 ± 3.47 , 34.95 ± 2.88 and $51.71 \pm 5.28\%$ (Table 2 and Figure 2). The coefficient percentage decreasing is statistically

Table 2 Median values of bevacizumab, total vascular endothelial growth factor and not bevacizumab-bound vascular endothelial growth factor before the 2nd and the 5th cycle of therapy

Time of blood withdrawal		PR	SD	PD
Before 2 nd cycle	BV (μg/mL)	2.61 ± 1.10	5.51 ± 5.28	2.33 ± 0.94
Before 5 th cycle	BV (μg/mL)	10.34 ± 2.72	3.98 ± 3.14	4.89 ± 1.98
	Ratio BV (5 th /2 nd)	3.96 ± 0.69	0.72 ± 0.25	2.10 ± 0.13
Before 2 nd cycle	VEGF basal (pg/mL)	376.39 ± 221.97	308.80 ± 177.76	395.98 ± 283.00
	VEGF total (pg/mL)	457.80 ± 46.84	310.18 ± 179.17	434.93 ± 12.90
	VEGF not BV-bound (pg/mL)	122.01 ± 18.54	165.67 ± 74.61	113.03 ± 25.75
	VEGF not BV-bound/VEGF total (%)	26.65 ± 1.33	53.41 ± 4.75	25.99 ± 5.23
Before 5 th cycle	VEGF total (pg/mL)	694.97 ± 19.17	304.61 ± 186.16	300.55 ± 74.28
	VEGF not BV-bound (pg/mL)	107.75 ± 27.08	106.46 ± 186.16	155.42 ± 31.68
	VEGF not BV-bound/VEGF total (%)	15.50 ± 3.47	34.95 ± 2.88	51.71 ± 5.28

BV: Bevacizumab; VEGF: Vascular endothelial growth factor; PR: Partial response; SD: Stable disease; PD: Progression disease.

Table 3 Vascular endothelial growth factor and angiopoietin-2 in colon cancer patients treated with FOLFOX regimen

	VEGF (pg/mL)		Ang-2 (pg/mL)	
	Total	After immunodepletion	Total	After immunodepletion
Patient No. 1	200.24	205.62	242.10	270.48
Patient No. 2	126.25	140.36	307.18	312.01
Patient No. 3	188.75	182.50	662.65	733.45
Patient No. 4	255.15	232.34	388.20	477.20

Ang-2: Angiopoietin-2; VEGF: Vascular endothelial growth factor.

significant in patients with PR and SD ($P < 0.05$), and its increasing is statistically significant in PD patients ($P < 0.05$). In particular, the ratio of not BV-bound VEGF to total VEGF before the 2nd cycle of therapy and before the 5th cycle decreased from 26.65% to 15.5% in PR group and from 53.41% to 34.95% in SD group. Conversely, in patients who showed a PD the ratio was higher than before the 2nd cycle, i.e., 51.71% vs 25.99% (Table 2 and Figure 2).

The analysis of Ang-2 levels conducted at the same time did not show any relation with either VEGF or therapy response. Only a slight, not statistically different reduction was found between the levels of Ang-2 before the 2nd and the 5th cycle of therapy, and no differences were evident among the three groups of patients (Figure 3).

DISCUSSION

To date, a number of different circulating biomarkers of response to antiangiogenic therapy have been investigated, including serum levels of pro-angiogenic factors such as VEGF, soluble VEGFR, collagen IV^[7,8] and not pro-angiogenic factors such as LDH and fibrinogen^[25], even if none have been validated for use in clinical practice. In particular, baseline concentration of serum VEGF (before BV treatment) has been proposed as a prognostic and/or predictive factor. However, no definitive results are available regarding VEGF modulation after BV therapy in CRC patients^[24]. In our study, we observed that the plasma levels of

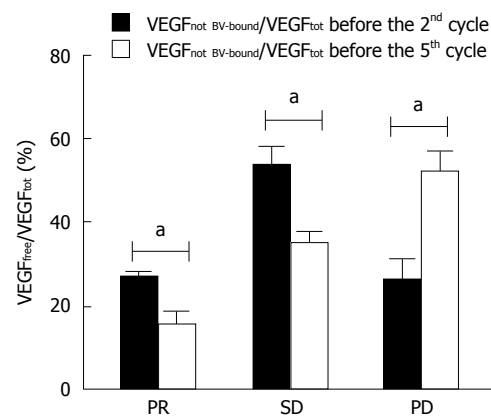


Figure 2 Residual not bevacizumab-bound vascular endothelial growth factor in plasma in function of time. Histograms represent the percentage of the ratio of not bevacizumab (BV)-bound vascular endothelial growth factor (VEGF) to total VEGF in plasma before the 2nd and the 5th cycle of therapy, respectively. ^a $P < 0.05$, the 2nd cycle vs the 5th cycle.

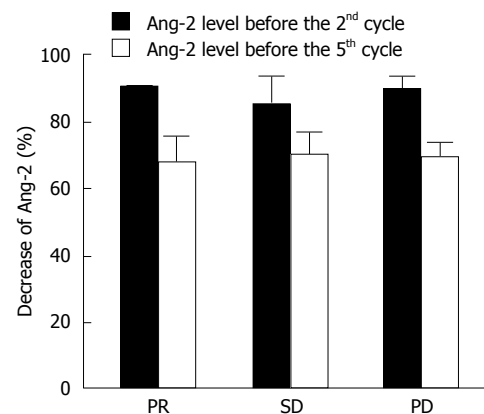


Figure 3 Decrease of angiopoietin-2 in plasma in function of time. The percentage of the decrease of angiopoietin-2 (Ang-2) level in plasma as respect the baseline level (before the beginning of therapy) is reported in function of timing after therapy, before the 2nd and the 5th cycle.

not BV-bound and total VEGF varies much fold among patients, with high standard deviations. Therefore we tried to normalize all values by performing the ratio between not BV-bound VEGF and total VEGF. As showed in Table 2, standard deviations of this ratio

expressed as percentage is lower than the absolute data. In particular, we saw that not BV-bound VEGF/total VEGF ratio significant decreased in patients with PR or SD during the treatment. At the same time, not BV-bound VEGF/total VEGF ratio in patients with PD before the 2nd cycle was similar to that of PR patients, but it strongly increased before the 5th cycle. In a comprehensive evaluation of total circulating VEGF-A in randomized phase III trials it was reported that short PFS and OS after BV treatment seemed to be associated with higher baseline circulating VEGF levels, even if the Authors excluded a predictive role for that angiogenic factor^[26]. Changes in VEGF concentration related to treatment have also been investigated. Keskin *et al*^[27] reported results of a preliminary evaluation of serum VEGF and basic fibroblast growth factor (bFGF) pre- and post-treatment levels as predictive of treatment response. In 33 mCRC patients treated with BV in combination with irinotecan-based chemotherapy, serum levels of VEGF and bFGF were higher than in healthy controls and pre-treatment low serum level of VEGF was associated with a significantly longer OS^[27]. Interestingly, previous studies showed a paradoxical increase of VEGF during BV treatment, probably due to assays that do not discriminate between not BV-bound and bevacizumab-bound VEGF. Loupakis *et al*^[13] reported that not BV-bound VEGF decreases after BV administration in 5 mCRC patients. Later, the same Authors confirmed that BV induce a prolonged and significant reduction of plasma active not BV-bound VEGF concentration in a larger cohort of CRC patients. Whereas, VEGF concentrations remained lower also at the time of PD suggesting other mechanisms of tumor resistance^[13].

Brostjan *et al*^[28] showed that the level of not BV-bound VEGF dropped significantly in 19 CRC patients treated with combination chemotherapy and BV in the neoadjuvant setting. In this study, VEGF level was measured in the plasma of all mCRC patients enrolled before starting therapy, and in agreement with results reported in literature no correlation was evident between the baseline amount of this factor and the response to the subsequent therapy regimen including BV^[28-31]. Furthermore, the comparison of VEGF before and after chemotherapy plus BV also showed an absence of correlation between this molecule and the response to therapy, in agreement with data from Loupakis *et al*^[13] study^[27].

The amount of BV in serum was also determined using a previously designed immunoassay^[23]. In agreement with a previous study analyzing BV pharmacokinetic in glioblastoma and breast cancer patients^[19], we showed that BV concentration was markedly increased in patients which achieved a PR than patients with SD and PD. In particular, Nogue *et al*^[19] demonstrate that low serum BV levels were associated with a PD, while high levels were associated with side effects. Comparison of median levels

confirmed that the concentrations were significantly different in this groups ($P > 0.05$) and serum BV levels could to be used as clinical pharmacodynamic/predictive biomarker.

Interestingly in this study, serum levels of BV, significantly higher in PR compared to SD and PD patients, suggest that lower values favor inefficacy of this drug. However, fold increase level of the drug in patients with PD was inconsistent with the idea of positive correlation between drug levels and therapeutic efficacy. A possible explanation for this could lie in the metabolism of BV. Recently, Panoilia *et al*^[32] analyzed the pharmacokinetics of BV and its relationship with VEGF in patients with mCRC receiving BV in combination with chemotherapy. They described a pharmacokinetic model that characterizes the *in vivo* interaction of BV with its soluble ligand, VEGF. Different levels of BV in patients were attributed to a possible role of VEGF polymorphisms. Although dosages of VEGF polymorphisms led no results in the kinetics alteration of BV, it would be interesting correlate different trends of BV serum in the different responses to treatment to the various polymorphisms of VEGF that could led to mechanisms of resistance to BV. In order to discover these mechanisms, we also analyzed the plasma level of Ang-2, hoping to highlight a correlation with clinical response and suggesting it as an additional predictive biomarker. Unfortunately, Ang-2 was not differently modulated in our samples in function of therapy response. Finally we have graphically represented the results of our study (Figure 4).

In conclusion, although basal VEGF level in the plasma of patients does not predict response to BV combined with chemotherapy, our data pointed out that the measurement of the ratio of not BV-bound VEGF to total VEGF could be relevant in monitoring the response to BV plus oxaliplatin-based chemotherapy. Moreover, this pilot study had the aim of finding a methodology which can standardize in all patients the response to anti-angiogenic therapy in function of VEGF and this ratio would appear to be very effective. An evaluation of these data in a larger series is advisable.

However, even if the high fold increased levels of BV in the serum of patients could play a role in determining the response to therapy, we have no pharmacokinetic data which emphasize the real predictive role of BV in mCRC. Hence, we suggest that both measurements of not BV-bound VEGF vs total VEGF and of BV serum levels should be taken into account while evaluating the clinical outcome of such patients.

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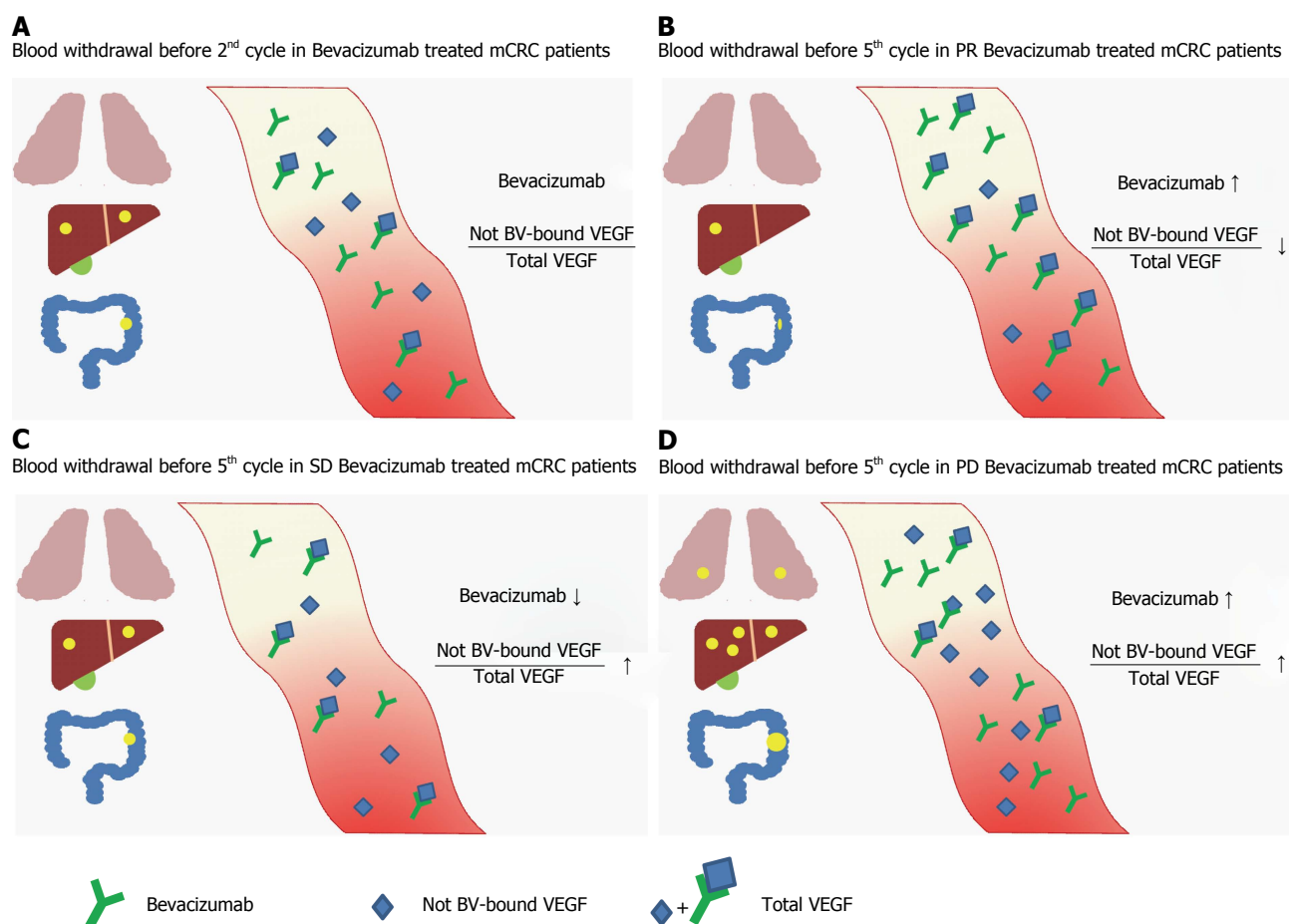


Figure 4 Grafical representation of bevacizumab not bevacizumab-bound vascular endothelial growth factor/total vascular endothelial growth factor before 2nd cycle in bevacizumab treated metastatic colorectal cancer patients (A) and before 5th cycle in partial response (B), stable disease (C) and progression disease (D) groups. BV: Bevacizumab; VEGF: Vascular endothelial growth factor; PR: Partial response; SD: Stable disease; PD: Progression disease; mCRC: Metastatic colorectal cancer.

COMMENTS

Background

To date, a number of different circulating biomarkers of response to anti-angiogenic therapy have been investigated, including serum levels of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), there is no evidence of predictive markers of response to bevacizumab (BV). Identification of predictive biomarkers would allow selection of patients most likely to benefit from treatment with BV, thereby avoiding toxicities. The identification of suitable biomarkers of response to BV is still an open question. The measurement of serum VEGF has been proposed as a predictive factor for this drug, even if literature data are contradictory.

Research frontiers

Baseline concentration of serum VEGF (before BV treatment) has been proposed as a prognostic and/or predictive factor. However, no definitive results are available regarding VEGF modulation after BV therapy in CRC patients.

Innovations and breakthroughs

The authors observed that the plasma levels of not BV-bound and total VEGF varies much fold among patients, with high standard deviations. Therefore they tried to normalize all values by performing the ratio between not BV-bound VEGF and total VEGF, the rediscovery of the predictive role of traditional biomarkers as not BV-bound VEGF/total VEGF plasma ratio together with the results of the bevacizumab pharmacokinetic in response, stable disease and progression settings of patients with metastatic colorectal cancer treated with bevacizumab plus oxaliplatin based chemotherapy supported our hypothesis.

Peer-review

Although the study has been done in a small subset of patents treated with chemotherapy plus bevacizumab in first and second-line therapy and it's exploratory it's interesting because shows that patients with response to therapy are those with a higher decrease of free VEGF (not bound to bevacizumab). The study is well-written and designed.

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Environmental risk factors for inflammatory bowel diseases: Evidence based literature review

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Abstract

AIM: To advances in genetics and immunology have contributed to the current understanding of the pathogenesis of inflammatory bowel diseases (IBD).

METHODS: The current opinion on the pathogenesis of IBD suggests that genetically susceptible individuals develop intolerance to dysregulated gut microflora (dysbiosis) and chronic inflammation develops as a result of environmental insults. Environmental exposures are innumerable with varying effects during the life course of individuals with IBD. Studying the relationship between environmental factors and IBD may provide the missing link to increasing our understanding of the etiology and increased incidence of IBD in recent years with implications for prevention, diagnosis, and treatment. Environmental factors are heterogeneous and genetic predisposition, immune dysregulation, or dysbiosis do not lead to the development of IBD in isolation.

RESULTS: Current challenges in the study of environmental factors and IBD are how to effectively translate promising results from experimental studies to humans in order to develop models that incorporate the complex interactions between the environment, genetics, immunology, and gut microbiota, and limited high quality interventional studies assessing the effect of modifying environmental factors on the natural history and patient outcomes in IBD.

CONCLUSION: This article critically reviews the current evidence on environmental risk factors for IBD and proposes directions for future research.

Key words: Environmental factors; Inflammatory bowel disease; Exosomes

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Core tip: Environmental factors are heterogeneous with varying effects during the life course of individuals with inflammatory bowel diseases (IBD). Studying the relationship between environmental factors and IBD may provide the missing link to increasing our understanding of the etiology and increased incidence of IBD in recent years with implications for prevention, diagnosis, and treatment. However, the impact of modifying specific environmental factors on causation and established disease remain poorly studied with limited high quality data from interventional studies to guide clinical practice.

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic idiopathic inflammatory bowel diseases (IBD). Although the exact pathogenesis of IBD remains unknown, part of the underlying mechanism is a deregulated host immune response to intestinal flora, in genetically susceptible individuals^[1,2]. The greatest risk for developing IBD is having a family history of the disease^[3]. The greatest risk is seen in monozygotic twins and if both parents suffer from IBD^[1]. The estimated relative risk to a sibling of a patient with IBD is 13-36 and 7-17 for CD and UC respectively^[2,4]. The first CD susceptibility gene, *NOD2* gene within the IBD 1 locus, was a major discovery in 2001^[2,3]. Hugot *et al*^[3] found independent associations with CD for three different polymorphisms of the *NOD2* gene. The risk of IBD varies depending on whether a subject has one copy (heterozygous) or both copies (homozygous) of the defective allele^[2]. However, genetic susceptibility does not completely explain the variance in disease incidence, suggesting a strong role for environmental factors^[3,4]. Environmental factors such as smoking, infection, drugs, stress, air pollution, water pollution, diet, and food additives have been investigated in IBD and other autoimmune diseases. These factors have been collectively referred to as exposomes^[5]. The term exposome refers to all possible environmental exposures on a human being from conception to death^[5]. Factors contributing to the exposome of humans are multifarious. Therefore, the study of the impact of exposomes on human health provides a complete view of human health and disease. Combining the study of exposomes with advances in genetics and immunology may unravel the etiology of diseases such as IBD, diabetes, and cancer, thus

Table 1 Commonly studied environmental factors in inflammatory bowel diseases

Environmental factor
Lifestyle
Smoking
Sleep
Stress
Diet
Breastfeeding
Pharmacologic agents
Non-steroidal anti-inflammatory drugs
Antibiotics
Oral contraceptives
Vaccination
Gut Microbiome
Dysbiosis
Ecological factors
Air Pollution
Water Pollution
Low Vitamin D
Surgery
Appendectomy

enabling the development of preventive interventions against specific exposomes^[6]. This article critically reviews the most commonly studied environmental risk factors associated with IBD and proposes directions for future research on environmental factors in IBD.

MATERIALS AND METHODS

A systematic literature search was conducted in PubMed, EMBASE, and Cochrane Library from 1965 through May 2016. The search terms: "environmental factors" and "inflammatory bowel disease"; "exposomes" and "IBD"; "environment" and "crohn's disease"; "environment" and "ulcerative colitis"; were used to identify relevant studies. In addition, bibliographies of the retrieved articles were searched to identify additional relevant articles on the most commonly studied environmental risk factors in IBD (Table 1). The 2009 Oxford Centre for Evidence-based Medicine (OCEBM) levels of Evidence (LOE) was used to assess the strength of evidence^[7] (Table 2). The 2009 OCEBM LOE was chosen because it evaluates what type of evidence is likely to provide the strongest support from studies assessing etiology, prevention, therapy, harm and prognosis without explicitly making definitive recommendations unlike GRADE which is intended for appraising systematic reviews used in developing guidelines^[7]. Moreover, OCEBM LOE can be applied in situations where there are no systematic reviews available^[7].

RESULTS

Hygiene

Strachan^[8] proposed the hygiene hypothesis in 1989 to explain the dramatic rise in atopic diseases. The central principle of this hypothesis is that abnormal immune responses such as autoimmunity and allergy

Table 2 Oxford Centre for Evidence-Based Medicine evidence levels of evidence scale

Level	Study questions on therapy/prevention, etiology/harm
1a	Systematic review (with homogeneity of RCTs)
1b	Individual RCT (with narrow confidence intervals)
1c	All or none studies
2a	Systematic review (with homogeneity) of cohort studies
2b	Individual cohort study (including low quality RCT, <i>e.g.</i> , < 80% follow-up)
2c	Outcomes research: ecological studies
3a	Systematic review (with homogeneity) of case control studies
3b	Individual case control study
4	Case-series (and poor quality cohort and case control studies ++)
5	Expert opinion without explicit critical appraisal or based on physiology, bench research or first principles

Users can add a minus-sign “-” to denote the level of evidence that fails to provide a conclusive answer because: either a single result with a wide confidence interval, or a systematic review with troublesome heterogeneity. Provided by Ref. [7].

are the result of improvements in personal hygiene and smaller family sizes which have reduced exposure to microbial stimulation^[8]. An expansion of this hypothesis is the “microflora” or altered microbiota hypothesis proposed by Noverr and Huffnagle and the IBD hygiene hypothesis^[9,10]. The microflora hypothesis proposes that changes in the gut microbiota due to dietary changes and antibiotic use in western countries have altered microbial mediated mechanisms of immunological tolerance^[9]. Taken together, these hypotheses suggest that environmental changes can impact the composition of gut microbiota and lead to disease^[8-10]. However, recent evidence suggests that the hygiene hypothesis is not applicable to all populations worldwide, and it may be most relevant in countries experiencing increasing affluence or following migration from resource poor to more affluent countries^[10].

Smoking

Cigarette smoking is the earliest environmental risk factor that has been consistently shown to be associated with IBD^[11-13]. The mechanism by which smoking exerts its effect in IBD is poorly understood. However, putative mechanisms by which smoking modulates the immune system in UC may involve the reduction in tumor necrosis factor (TNF alpha) production *via* the action of nicotine on the nicotinic acetylcholine receptor $\alpha 7$ subunit, increased production IL-10 in response to carbon monoxide in cigarette smoke, increased mucin synthesis, decrease in IL-8 expression, hypoperfusion of the rectum and acutely damaged colonic tissue^[12]. In CD, increased carbon monoxide from cigarette smoke may cause impairment in vasodilation capacity in chronically inflamed micro vessels, resulting in ischemia, and perpetuating ulceration and fibrosis^[12]. Decreased

total radical-trapping antioxidant potential and abnormalities of the microvasculature, and a defect in bacterial clearance or macrophage deficiency may also play a role^[12].

Smoking and Crohn’s disease risk of disease:

Smoking increases the risk of developing CD among current smokers [HR = 1.90 (95%CI: 1.42-2.53)]^[11]. The increased risk is associated with the number of pack years smoked (*P* trend < 0.0001), whereas smoking cessation is associated with a reduction in the risk [HR = 1.35 (95%CI: 1.05-1.73)]^[11]. Passive smoking exposure in childhood is no longer considered a risk factor for incident CD^[14] (LOE 3A, 2B).

Risk of disease progression: Smoking increases the risk for advanced and difficult to treat disease; It increases the risk of penetrating intestinal complications, strictures or fistulae, and need for surgical resections (first or second surgery)^[13,15,16]. Smoking cessation results in decreased risk of CD, decreased risk of flares, decreased need for steroids and immunosuppressive therapy (IST)^[13,17-19] (LOE 3b, 2b).

Risk of relapse: Current smoking is associated with higher relapse rates^[13,15,17]. Smoking cessation is associated with a 32% reduction in the risk of a relapse as compared with continued smokers^[13]. Smoking cessation can be achieved in IBD patients by utilizing appropriate counselling services, nicotine replacement therapy and pharmacologic agents such as bupropion and varenicline^[12,18,19] (LOE 2b).

Smoking and ulcerative colitis risk of disease: In contrast to CD, current smoking is protective against UC, however the mechanism by which smoking exerts its protective effect in UC has not been clearly defined^[12]. A case-control study of the effect of smoking on the risk of acquiring UC among 212 individuals showed that the relative risk of UC among former smokers increased in proportion to the cumulative number of cigarettes smoked before the onset of disease, suggesting a causal relationship between smoking and disease occurrence^[20]. Analysis of 400 incident cases from the nurses’ health study showed that current smoking had a protective effect on the development of UC [multivariate HR = 0.86 (95%CI: 0.61-1.20)], whereas smoking cessation increased the risk of UC [HR = 1.56 (95%CI: 1.26-1.93)]^[11]. The risk of UC was significantly increased 2-5 years after smoking cessation [HR = 3.06 95%CI: (2.00-4.67) and remained elevated over 20 years^[11] (LOE 2b, 3a).

Risk of disease progression: Current smoking is associated with benign disease course, low hospitalization rates, decreased need for steroids in UC suggesting a less severe clinical presentation and

a better long term prognosis than in nonsmokers^[21]. However, both hospitalization and colectomy occurred more frequently among smokers who quit before disease onset^[22]. Furthermore, hospitalization and colectomy occurred most frequently in the heaviest smokers who quit before disease onset^[22].

Risk of relapse: Current smoking is associated with lower relapse and colectomy rates than nonsmokers^[22-24]. Overall, there is robust evidence from observational studies and meta-analyses on the association of smoking with IBD. Although smoking has been identified as a modifiable environmental risk factor for IBD, the specific mechanism by which it exerts its effect in UC and CD remains unclear (LOE 3b, 2b).

Environment, sanitation, industrialization and socio-economic status

Public health strategies such as vaccination and environmental sanitation as well as the increasing use of antibiotics has led to changes in the interaction between humans and microbes in the environment. Consequently, improvements in hygiene and health care can alter the composition of the gut microbiota and lead to a state of disequilibrium between protective and pathogenic bacteria (dysbiosis)^[8,9].

Risk of disease: A population based cohort study of 2144660 Canadian immigrants showed that younger age at arrival to Canada was associated with increased risk of IBD in immigrants^[25]. Canadian-born children of immigrants from Sub-Saharan Africa, Middle East/North Africa, South Asia, North America/Western Europe had a similar risk of IBD as children of nonimmigrants^[25]. However, the incidence of IBD remained lower among children of immigrants from East Asia and the Pacific, indicating that the underlying risk is activated with earlier life exposure to the Canadian environment in certain groups^[25]. A systematic review of case-control and cohort studies found a positive association between urban environment and both CD and UC [pooled IRRs for urban vs rural environment for UC and CD studies were 1.17 (1.03, 1.32) and 1.42 (1.26, 1.60), respectively], however differences in study design, study quality and lack of data from low prevalence regions (*i.e.*, developing world) limit the generalizability of the results^[26]. Data on the association of socio-economic status and risk of IBD is mixed; a population-based study from Canada reported that IBD patients are not of a higher socioeconomic status^[27]. A study from France reported that the relative risk (RR) for CD and UC was higher in rural and peri-urban areas with no association with socio-economic status^[28] while another study from France reported that RR of CD was higher in urban areas and areas with poor sanitary

equipment^[29]. However, no significant association was found between socioeconomic variables and incidence of UC^[29]. Recent data from China, a rapidly industrializing nation with a historically low prevalence of IBD^[30] showed that the incidence of CD was significantly higher in affluent areas than less affluent areas suggesting that high socio-economic status was associated with increased incidence of IBD^[31]. Data are lacking on the effect of urbanization and socio-economic status on the risk of disease progression and relapse (LOE 2a, 2b, 2c).

Air pollution

Air pollution has dramatically increased in recent years, particularly in developing countries in Asia that are experiencing rapid industrialization and the highest increase in IBD incidence^[31,32]. Exposure of the gut to air pollutants can occur *via* inhalation of gaseous pollutants, mucociliary clearance of particulate matter (PM) from the lungs and contamination of food and water sources (LOE 4, 2c).

Risk of disease: Kaplan *et al.*^[32] showed that residential exposures to Sulphur dioxide (SO₂) and Nitric oxide (NO₂) may increase the risk of early-onset UC and CD respectively. It has been hypothesized that the effect of air pollution and PM on the incidence of IBD is mediated by the intestinal microbiota; however, conclusive scientific evidence is lacking^[33] (LOE 2c, 5).

Risk of disease progression: Ananthakrishnan *et al.*^[34] showed that an increase in the density of pollutant emission by 1-log was associated with a 40% increase in the rate of IBD hospitalizations (incidence RR = 1.40; 95%CI: 1.31-1.50) for both UC and CD hospitalization). Data are lacking on the effect of air pollution on relapse in patients with quiescent IBD (LOE 2c).

Water pollution

Risk of disease: Ingestion of pollutants and PM *via* water sources may induce systemic effects that may impact the incidence, frequency of flares, and success of therapy in IBD^[35-38]. Antagonists of steroid receptors that may interfere with treatment have been found in bottled water^[38]. Endocrine-disrupting chemicals (EDCs) such as phthalic acid (used as plasticizers for polyvinyl chloride, polystyrene, and many other polymers) and nonylphenols (used in manufacturing antioxidants, lubricating oil additives, laundry and dish detergents, emulsifiers, and solubilizers) may modify glucocorticoid action by altering steroid hormone metabolism through the pregnane X receptor (PXR)^[35]. EDCs can influence several steroid receptor proteins such as the glucocorticoid receptor, androgen receptor, AHR, peroxisome proliferator-activated receptor gamma (PPAR-γ) as well as cytochrome P450 enzymes^[35].

In-vitro studies suggest that the glucocorticoid receptor and PPAR- γ may play an important role in the pathogenesis of IBD^[35-37]. However, there are no supporting *in-vivo* studies on the effect of EDCs on the risk of incident IBD, disease progression and relapse (LOE 5).

Vitamin D

Vitamin D has immuno-regulatory properties in several autoimmune diseases *via* its genomic actions on the vitamin D receptor (VDR)^[39-41]. There is accumulating evidence that vitamin D may play an integral role in the incidence and disease activity in IBD^[42-62] (LOE 5).

Risk of disease: Vitamin D3 (1, 25(OH) D3) decreases production of regulatory Th17 cells and influences the function of natural killer T cells^[41,45]. Experimental studies in VDR and IL-10 knockout mice have shown increased expression of inflammatory cytokines in the colon and increased susceptibility to experimental models of colitis^[43,46,47]. Subsequent administration of 1,25(OH)2D3 ameliorated colitis and suppressed tumor necrosis factor alpha-related gene expression in the colon of the mice^[43,47,61]. However, genome-wide association studies (GWAS) of VDR polymorphisms and IBD have yielded conflicting results; some studies support a positive association while others do not^[50,53,58]. Epidemiologic data suggests that the incidence of IBD is higher in residents of northern latitudes compared with southern latitudes, possibly explained by differences in ultraviolet light exposure^[42,44,49,55]. However, other studies report no geographic/latitudinal difference^[42]. Evidence from the Nurses' Health Study showed that women in the highest quartile of predicted vitamin D level had a 40% reduction in the risk of CD over 22 years of follow-up compared with those in the lowest quartile (HR = 0.54, 95%CI: 0.30-0.99)^[42]. However, there was no effect on the risk of UC^[42] (LOE 2a, 3b).

Risk of disease progression: Evidence in support of the association of vitamin D deficiency with greater disease activity is more compelling^[48,51,52,57,59,60]. Two small open label studies showed that supplementation with Vitamin D was associated with lower CDAI scores^[51,60]. Recently, a large prospective cohort study showed that low vitamin D was associated with higher morbidity and disease severity in patients with UC and CD^[59] (LOE 2b).

Risk of relapse: One RCT showed that Vitamin D supplementation was associated with a reduced risk of relapse over the subsequent 12 mo of follow-up compared with those receiving placebo^[51]. A meta-analysis of 14 case-control studies showed that IBD was significantly associated with having higher odds of vitamin D deficiency^[44].

On balance, the effect of vitamin D on the in-

cidence of IBD is unclear. Vitamin D deficiency has been associated with several chronic diseases and the association with IBD is not causal^[54,56]. What we do know is that Vitamin D deficiency is common in IBD patients and long-standing deficiency has been associated with reduced bone mineral density^[56]. There is robust evidence that deficiency is associated with disease activity^[55-59] and vitamin D supplementation appears to improve CDAI and QOL^[51,52,57,60]. Large, prospective, double-blinded RCTs are necessary to define the role of vitamin D therapies in prevention and treatment of IBD. However, in the absence of such studies it is prudent to screen for and treat vitamin D deficiency or insufficiency in patients with IBD (LOE1a, 2a).

Gut microbiome

The gut microbiome consists of a vast number of microorganisms belonging to over 1000 species^[63]. The human gut is sterile at birth; bacterial colonization occurs within the first hours of life and increases in number and diversity, depending on the mode of delivery, type of infant feeding, and environmental exposure^[64]. Microbial diversity quickly increases in early childhood and leads to the development of the adult gut microbiome^[64]. Composition of the gut microbiota is influenced by numerous factors such as antibiotic use, host genetics, diet, phylogeny of the host and intestinal inflammation^[64-69]. The majority of the bacteria in the adult gut belong to three phyla, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*^[65]. Over time, humans have co-evolved with gut microbes to exist in a symbiotic relationship. The human gut provides the optimal environment for the microbiota to thrive while the microbiota provide physiological benefits such as fermentation of indigestible carbohydrates; synthesis of short chain fatty acids (SCFA) and certain vitamins; biotransformation of conjugated bile acids; degradation of dietary oxalates and resistance to colonization by pathogenic micro-organisms^[66,67]. Studies have demonstrated that the gut microbiota have a significant effect on the development of the immune system^[68,69] (LOE 1a, 2b, 5).

Risk of disease: Animal studies have shown that bacterial colonization of the gut is critical for the development of intestinal inflammation in IBD^[68,69]. Additionally, GWAS have identified 163 genetic risk loci associated with IBD, including 28 shared between CD and UC^[2,70]. Although host genetics play a critical role in disease pathogenesis, non-genetic factors also play a substantial role in the development of IBD^[70]. Many of the genetic risk alleles associated with IBD are involved in regulation of the innate or adaptive immune system^[2,70]. Taken together, the accruing evidence supports the notion that IBD is due to an abnormal immune response to microbial stimulation in genetically susceptible individuals^[68-71].

Clinical observations also support the role of gut microbiota in IBD^[72-74]. First, IBD typically affects intestinal regions with the highest concentration of bacteria and the use of antibiotics can be effective in the management of IBD^[73]. Second, studies of fecal diversion revealed recurrence of colitis with reintroduction of the fecal stream^[73,74]. Furthermore, significant alterations of the gut microbiota have been associated with IBD, leading to the notion that dysbiosis leads to disease pathogenesis^[72,75-77]. A case control study using metagenomic analysis of microbial community structure showed a statistically significant difference in temporal stability and microbial diversity between the microbial compositions of IBD patients and non-IBD controls^[77]. Furthermore, the authors reported that microbiota of IBD patients displayed a reduction in the levels of two phyla of bacteria, *Firmicutes* and *Bacteroidetes*, compared with non-IBD controls^[77]. New sequencing technologies may provide opportunities to increase our understanding of how dysbiosis may affect IBD^[77-79]. However, factors that trigger this change in the delicate balance of the gut microbiota are poorly understood. Prospective studies are required to determine if the loss of certain classes of bacteria can predict individuals with a greater risk of developing IBD (LOE 1a, 3b, 4, 3b, 5).

Risk of disease progression: Dysbiosis has been associated with active disease and relapse in a limited number of studies. Sokol *et al.*^[80] demonstrated that *Fecalibacterium prausnitzii* (Firmicutes) and *Bifidobacteria* are underrepresented in patients with active IBD and infectious colitis. Reduction in the numbers of *F. prausnitzii* was associated with a higher risk of postoperative recurrence of ileal CD^[81]. Lower numbers of *F. prausnitzii* on resected ileal mucosa of CD patients was associated with endoscopic recurrence at 6 mo^[81]. A decrease in *F. prausnitzii* was also associated with the time to relapse after infliximab withdrawal in another study^[82]. In patients with UC, quantities of different species of lactobacillus were significantly lower in patients with active inflammation compared with patients in remission^[83]. These studies imply that a reduction in intestinal *F. prausnitzii*, *Bifidobacteria*, and *Lactobacillus* species may be important in the initiation of CD or UC respectively (LOE 2b).

Risk of relapse: A cohort study of CD patients demonstrated lower rates of *Firmicutes* in relapsers compared with non-relapsers^[82]. A low rate of *F. prausnitzii* and a low rate of *Bacteroides* independently predicted relapse in CD patients^[82]. Furthermore, a decrease in *Firmicutes* was shown to correlate with the time-to-relapse after infliximab withdrawal^[82]. A cohort study of UC patients showed that low levels of *F. prausnitzii* was associated with a four-fold increase

in the risk of relapse^[84]. The recovery of the *F. prausnitzii* population after relapse was associated with maintenance of clinical remission in a cross-sectional study of UC patients^[84] (LOE 2b).

Infection

The gut bacterial composition of patients with IBD significantly differs from that of non-IBD controls^[78,80,81]. However, studies to identify microbial pathogens that cause IBD has met with limited success^[70]. Although various pathogenic organisms have been suggested, no pathogen has been consistently implicated in IBD^[70]. Pathobionts are symbiotic organisms within the gut that typically do not elicit an inflammatory response, however, under specific environmental conditions, pathobionts have the potential to cause inflammation leading to disease^[70,76]. This has led to the notion that the etiologic agents for dysbiosis in IBD patients are not necessarily pathogens, but rather disproportionate populations of pathobionts^[70,75,76]. Animal and human studies have shown that deficiencies in T-regulatory-cell (T-Reg-cell) populations or function are central to the pathogenesis of rheumatoid arthritis, asthma, type 1 diabetes and IBD^[85,86]. Pro-inflammatory TH17 cells have been shown to antagonize FOXP3+ Treg-cells and the numbers and function of certain TReg-cell populations are reduced in germ-free animals^[87,88]. Dysbiosis involves an abnormal change in the composition of the gut microbiota whereby either the numbers of symbionts are reduced and/or pathobionts are increased. The various causes for this change in microbiota are not entirely clear, however, the result is non-specific gut inflammation, which can trigger IBD in genetically susceptible individuals. This gut inflammation can be exacerbated by opportunistic organisms such as viral, bacterial and fungal infections. Thus, prior infections may lead to chronic IBD in patients with a genetic predisposition while coexisting IBD may predispose patients to enteric infections (LOE 3b, 5).

Role of Bacterial infections: Bacterial infections have been implicated as triggers of relapse in IBD^[89-95]. *Clostridium difficile* infection (CDI) in patients with IBD is associated with significant morbidity^[90,93]. Hospitalized patients with CDI-IBD have a 4-fold greater mortality risk than patients who do not have IBD-CDI^[89,93]. However, there is no evidence to suggest that prior CDI predisposes patients to the development of IBD. Enteric infections such as adherent-invasive *E-coli*, *Salmonella* and *Campylobacter*, *Mycobacterium avium* species have been hypothesized to increase the risk of development of CDI and IBD^[70,91,92,94]. However, the apparent increase in IBD risk after enteric infections, particularly in the year after the diagnosis of infection, may be due to a detection bias (LOE 2c, 2c, 5).

Role of viral infections: Cytomegalovirus, ebstein barr virus and human herpes virus have been implicated in exacerbations of IBD or superimposed infection in IBD^[96-107]. Human papillomavirus has been linked with squamous cell carcinoma in patients with IBD^[98]. However there is no conclusive evidence of a causal link between viruses and IBD. In a recent study using metagenomic analysis, patients with herpes viridae sequences in their colon demonstrated increased expression of human endogenous viral sequences and differences in the diversity of their microbiome^[106]. Although the study provided a promising approach to better understand virus-host and phage-bacteria interactions in IBD, further studies are needed to define the contribution of viral infections in the etiopathogenesis of IBD (LOE 3b, 3c, 4, 5).

Role of Parasitic infections: Parasites such as helminths are thought to play an immunomodulatory role in IBD and loss of helminth infections has been proposed as a possible explanation for the reduced incidence and prevalence of IBD in developing countries; the "IBD hygiene hypothesis"^[108]. Animal, clinical and epidemiological studies support this hypothesis and several distinct immuno-regulatory mechanisms have been described^[108-112]. Helminths promote IL10 secretion, a regulatory cytokine that down regulates Th1 responses and colitis in murine models of IBD^[110]. Helminths increase the number of Treg-cells in MLNs and the intestinal lining, and promote lamina propria T cells to make more IL10 and TGF β ^[112]. Helminths promote the growth of IL4-producing, Th2 cells which inhibit Th1 responses supporting the importance of worm-induced Th2 cytokines for disease control^[109]. Helminths have also been shown to increase the number of Treg-cells that help maintain the gut lining in a state of immune tranquility and limit the potential for IBD^[111,112]. Recently, an elegant experimental study showed that helminth infection protected mice deficient in the CD susceptibility gene Nod2 from intestinal inflammation by inhibiting colonization with *Bacteroides* species via a TH-2 dependent pathway, which promoted the establishment of protective microbiota enriched in *Clostridiales*^[109]. Additionally, that study showed that individuals from helminth-endemic regions harbored a similar protective microbiota, and that deworming treatment reduced *Clostridiales* and increased *Bacteroidales*, further supporting the role of parasitic infection in the IBD -hygiene hypothesis whereby certain individuals are genetically susceptible to changes in the gut microbiome^[109].

One RCT assessed the safety and efficacy of modified *Trichuris Suis* in patients with CD and reported no significant treatment-related side effects^[113]. A subsequent systematic review concluded that there was insufficient evidence to allow any firm conclusions

regarding the efficacy and safety of helminths for treating IBD^[114]. Further RCTs are required to assess the efficacy and safety of helminth therapy in IBD.

Diet

The human diet is influenced by environmental and cultural practices. Diet can influence intestinal inflammation via several pathways, such as, altering the gut microbiome, affecting gastrointestinal permeability, and direct effect of dietary constituents acting as food antigens^[115] (LOE 5).

Risk of disease: Evidence from animal^[116,117] and epidemiologic studies^[115,118] suggests that dietary factors play an important role in gut inflammation and the risk of developing IBD (LOE 2b,3a,5).

Fats

Risk of disease: A systematic review of 19 studies (18 case-control and one cohort; $n = 2609$; 1340 UC and 1269 CD patients) reported increased risk of developing UC with high intake of total fat, polyunsaturated fatty acid (PUFAs), omega-6 fatty acids, and increased risk of CD with high intake of PUFAs, omega-6 fatty acids, saturated fats^[118]. Consumption of high-fat diet worsened dextran sodium sulfate-induced colitis in mice, possibly by increasing colonic epithelial non classical natural killer T cells, and reducing circulating Treg-cells^[117]. A high saturated fat diet altered bile acid composition, and increased expansion of sulfate-reducing bacteria (*Bilophila wadsworthia*), which in-turn can produce greater amounts of mucosally toxic hydrogen sulfide and induce colitis in IL-10 deficient mice^[116]. The results of these animal studies have not been translated to humans (LOE 2b, 5).

Fiber

Risk of disease: A study using IL-10 deficient mice showed that consumption of soluble fibers reduced intestinal inflammation^[119]. In humans, high intake of dietary fiber, particularly fruits and cruciferous vegetables was associated with decreased risk of CD, but not UC (HR = 0.59; 95%CI: 0.39-0.90)^[120]. The protective effect of fiber was observed to be statistically significant in those consuming more than 22.1 g/d in another study^[118]. Additionally, high intake of fruits was associated with a 73%-80% decreased risk of CD in the same study^[118] (LOE 2b, 2b, 5).

Risk of disease progression: *Plantago ovata* seeds (soluble fiber) were shown to have anti-inflammatory activity in HLA-B27 transgenic mice^[121]. This animal model was further tested in a RCT that showed that *Plantago ovata* may be as effective as mesalamine for maintenance of remission in patients with UC^[122]. However, the result of that study has not been validated

by other RCTs (LOE 1b, 2b).

Carbohydrates Risk of disease: A systematic review showed no consistent association between total carbohydrate intake and IBD risk, even in studies reporting greater than double the recommended daily intake (130 mg total carbohydrates per day)^[118] (LOE 3a).

Animal protein (red meat, processed meat, poultry, dairy products)

Risk of disease: The association between high meat intake and IBD is unclear, majority of studies show a positive association of total protein intake and IBD (87% to 148% increased risk)^[118]. However, the association between meat intake and risk of IBD was statistically significant in only two studies (LOE 2b).

Risk of disease progression and relapse: The amino acid tryptophan supplies a crucial intermediate metabolite for the action of aryl hydrocarbon receptor (AhR) which suppresses immune responses in dendritic cells. A deficiency in AhR increases production of proinflammatory Th17 cells^[123,124]. High tryptophan availability causes lactobacilli to switch their metabolism and produce an AhR ligand-indole-3-aldehyde that contributes IL-22 induction and subsequent IL-22 mediated attenuation of colitis^[123]. Similarly, indole-3-carbinol present in fruits and vegetables (such as cauliflower, broccoli, cabbage), activates the AhR and ameliorates colitis in mice^[120]. Thus animal proteins may modulate inflammation in IBD *via* the actions of specific amino-acids or their metabolites on immune function. However, diet-based interventions to maintain remission in CD and UC have shown limited benefit in maintaining remission or preventing relapse^[125-128] (LOE 1b, 3b, 5).

Food antigens

Risk of disease: Food antigens may act as important stimuli of the mucosal immune system leading to the pathogenesis of IBD. Patients with IBD report intolerance to several food items^[125]. However, the abundance of different potential food antigens and lack of high quality evidence on the effect of specific food antigens on the risk of disease in IBD make the association difficult to characterize (LOE 4).

Risk of disease progression and relapse: The role of food antigens on disease progression was studied in 40 patients with CD^[129]. Food specific IgG4 levels were used to select which foods to exclude in the intervention diet. The daily stool frequency significantly decreased by 11% in patients randomized to the intervention diet compared with the sham diet^[129]. Additionally, patients on the intervention diet had reduced abdominal pain and improved general well-being^[129]. The results of this study should to be

interpreted with caution because of the presence of several confounders and a high dropout rate.

Food additives

Risk of disease: Food additives such as Aluminum, titanium dioxide (TiO₂), and Microparticles/nanoparticles have been implicated in murine models of colitis^[130,131]. Aluminum is a component of several processed foods, toothpaste, deodorants, and cosmetics. Oral administration of aluminum at toxic levels worsened intestinal inflammation in mice with 2,4,6-trinitrobenzene sulfonic (TNBS) acid- and dextran sodium sulfate (DSS)-induced colitis and chronic colitis in interleukin 10 deficient mice^[131]. Aluminum impaired intestinal barrier function and enhanced intestinal bacterial translocation, favoring development of granulomas in mice^[131] (LOE 5, 5).

Risk of disease progression and relapse: Microparticles are used as food additives, anticaking agents, or food colorants and accumulation of microparticles have been demonstrated in Peyer's patches^[130]. NLRP3 is a multiprotein complex containing caspase-1, which activates the proinflammatory cytokines IL-1b and IL-18. TiO₂ particles were shown to activate the NLRP3 inflammasome^[130]. Thus, TiO₂ can be absorbed by intestinal epithelial cells and may aggravate inflammation in susceptible individuals^[130]. The aforementioned evidence suggests that food additives and microparticles may worsen intestinal inflammation and contribute to disease progression or relapse in individuals with IBD (LOE 5).

Diet and the gut microbiome: There is increasing evidence demonstrating an association between diet and the gut microbiome^[132-136]. Analysis of fecal 16S rRNA sequences from 60 mammalian species revealed clustering according to diet (herbivore, carnivore, and omnivore) and host phylogeny^[135]. The functional evolution of the gut microbiome in relation to the human diet was demonstrated in another study by using shotgun metagenomics sequencing^[134]. Differences in microbial genes that encode for enzymes involved in carbohydrate and amino acid metabolism have been demonstrated between herbivores and carnivores^[136]. This suggests that long-term co-evolution of humans and gut microbiota has modified the composition of the human gut microbiome^[132] (LOE 4).

Risk of disease: De Filippo *et al.*^[132] examined the fecal microbiome of Italian children (age 1-6 years) compared to that of children (age 1-6 years) from rural sub-Saharan Africa (Burkina Faso) and showed that there were similarities in the genera of gut bacteria present in the gut among children aged 1-2 years from both groups, possibly explained by breast feeding. However, there were considerable differences

in the gut microbiome between the African children, fed a traditional high-fiber diet, and the European children, fed a modern Western diet among children older than 2 years^[137]. African children showed a significant enrichment in *Bacteroidetes* and depletion in *Firmicutes* ($P < 0.001$), with abundance of bacteria from the genus *Prevotella* and *Xylanibacter* (known to contain a set of bacterial genes for cellulose and xylan hydrolysis), completely lacking in the European children. In addition, significantly more SCFAs ($P < 0.001$) were found in African children than European children. Also, *Enterobacteriaceae* (*Shigella* and *Escherichia*) were significantly underrepresented in African children compared with European children ($P < 0.05$)^[132]. Wu *et al.*^[136] demonstrated that long-term agrarian dietary patterns are associated with an enterotype dominated by *Prevotella*, a genus frequently observed in people from rural Africa, underscoring the impact of diet on the microbiome in healthy human patients. Wu *et al.*^[136] also demonstrated that a long-term diet high in animal protein and fats and low in carbohydrates, similar to a “Westernized” diet, is associated with high quantities of *Bacteroides* and low quantities of *Prevotella*, further supporting the impact of diet on the gut microbiome. Further studies on the link between diet, gut microbiota, and the development of IBD are needed to provide important insights into the association of a “westernized diet” with the increasing incidence of IBD (LOE 4).

Non-steroidal anti-inflammatory drugs

Cyclooxygenase (COX) is an important enzyme that is found in two isoforms in the body COX-1 and COX-2. COX-1 is present at constant levels in some tissues, whereas COX-2 is an inducible enzyme that can be up regulated by inflammation. COX-1 produces prostaglandins (PG) in the intestine to maintain the gut epithelial barrier and COX-2 mediates inflammation. Prostaglandins (PGs) promote inflammation, mucus production, and vascular flow in the gut. non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2, whereas COX-2 inhibitors selectively inhibit COX-2.

Risk of disease: Inflammation of the colon leads to an increase in PG synthesis and upregulation of COX-2. Thus it would seem logical that COX-2 inhibition could have a protective role in IBD. However, the role of other mediators has not yet been defined and the true effect of COX-2 inhibition has not been fully elucidated. It can also be argued that since COX-1 protects the gut epithelium, NSAIDs could cause or worsen IBD by breaking down the barrier between the immune system and intestinal luminal antigens. However, no causal relationship between NSAID use and incident IBD has been established.

Risk of disease progression: High-dose NSAIDs have been associated with an increase in disease activity in patients with CD or UC, while low-doses of NSAIDs were not associated with a higher disease activity index (DAI) score among CD patients^[137]. In contrast, another study reported no association between NSAID use and increased disease activity in IBD, suggesting that NSAID use in IBD deserves further study before recommending that patients refrain from their use under all circumstances^[138].

Risk of relapse: Nonselective NSAIDs were associated with a 17%-28% relapse rate within 9 days of ingestion in patients with quiescent IBD^[139]. In another study, the adjusted odds ratio between NSAID use and relapse was 6.31 (95%CI, 1.16-34.38, $P = 0.03$)^[140]. Similarly, Cox-2 inhibitors have been associated with relapse in patients with CD and UC suggesting that the all classes of NSAIDs are associated with relapse in IBD patients^[141,142]. However, other studies have reported no increase in flares and a beneficial safety profile during short-term treatment of IBD-associated arthritis and arthralgia^[143,144] (LOE 2b, 4).

Antibiotics

Risk of disease: Antibiotic use can induce selection pressure and alter the gut microbiome^[145-147]. A case-control study showed that treatment for pneumonia before the first 5 years of life increased the risk for childhood and adult-onset CD^[145]. Another study found that individuals diagnosed with IBD were more likely to have been prescribed antibiotics 2-5 years before their diagnosis^[146]. A recent meta-analysis showed that exposure to antibiotics was significantly associated with newly diagnosed CD (OR = 1.74, 95%CI: 1.35-2.23) but not UC (OR = 1.08, 95%CI: 0.91-1.27)^[147] (LOE 2a, 3b, 3b).

Risk of disease progression: In a systematic review of RCTs, antibiotics were superior to placebo at inducing remission in patients with active CD (RR of CD not in remission = 0.85; 95%CI: 0.73-0.99, $P = 0.03$)^[71]. Antibiotics were also superior to placebo for reducing fistula drainage in CD patients with perianal fistulae (RR = 0.8, 95%CI: 0.66-0.98)^[71]. In active UC, antibiotics were superior to placebo for inducing remission (RR of UC not in remission = 0.64, 95%CI: 0.43-0.96)^[71].

Risk of relapse: Antibiotics were superior to placebo (RR of relapse = 0.62, 95%CI: 0.46-0.84) for preventing relapse in patients with quiescent CD^[71]. Nitroimidazoles (metronidazole and ornidazole) have been shown to be effective in preventing post-operative recurrence of CD^[148,149]. The evidence on the effect of antibiotics on disease activity and relapse in IBD is limited because a diverse number of antibiotics

with different spectra of activity were grouped together making interpretation and generalizability difficult. However, despite the absence of robust data, antibiotics are widely used therapy in IBD.

Oral contraceptives

Risk of disease: Oral contraceptive pills (OCPs) were positively associated with UC and CD in a meta-analysis of 14 case control studies^[150]. The pooled RR for women currently taking OCPs was 1.46 (95%CI: 1.26-1.70, $P < 0.001$, adjusted for smoking) and 1.28 (95%CI: 1.06-1.54, $P = 0.011$, adjusted for smoking) for CD and UC respectively^[150]. The risk of CD was greater with prolonged exposure to OCPs and women who discontinued OCPs were no longer at a significantly increased risk for CD^[150]. Similarly, OCPs were shown to increase the risk of UC and CD in another study, however the risk of UC was only increased in patients with a history of smoking, suggesting that smoking was a confounding variable^[151]. Hormone replacement therapy (HRT) in post-menopausal women was shown to increase the risk of UC but not CD^[152].

Risk of disease progression: A case control study showed that OCPs have no effect on disease activity in CD^[153]. In contrast, Kane *et al.*^[154] showed that HRT was protective against disease activity in post-menopausal women with IBD (HR = 0.18, 95%CI: 0.04-0.72). A dose-response effect was noted with longer duration of HR, however the results should be interpreted with caution because it was a small single center retrospective study with limited generalizability.

Risk of relapse: A prospective cohort study showed that women who continued to take OCPs were at a threefold increased risk of developing a relapse of CD; this effect was stronger among women who were prescribed OCPs and smoked, suggesting that smoking was a confounding variable^[155]. The mechanism by which OCPs increase the risk of IBD is unknown, estrogen enhances humoral immunity and proliferation of macrophages, while progesterone suppresses immune responses^[156]. Therefore, it is conceivable that estrogen enhances inflammation and progesterone suppresses inflammation in patients with IBD.

Stress

Stress is defined as a state of disharmony or threatened homeostasis^[157]. The hypothalamo-pituitary-adrenal (HPA) axis and the immune system work closely together when the body is confronted with a stressful response. When stimulated by a stress event, the immune system activates the HPA axis by producing cytokines that ultimately result in the production of powerful anti-inflammatory agents such as glucocorticoids^[157]. Disruptions of the HPA axis and immune

system loop could potentially lead to diseases with an inflammatory and behavioral component due to abnormal responses to stressful stimuli. The loop that connects the immune system to the HPA is complex and disruptions at different levels could lead to different manifestations of disease^[158].

Risk of disease: A few studies have shown that stress is associated with increased relapse in patients with UC and CD^[159,160]. However, there is no evidence that stress is associated with increased risk of incident IBD (LOE 2b).

Risk of disease progression: An interventional study evaluated the effects of the Breath-Body-Mind Workshop (BBMW) (breathing, movement, and meditation) vs an educational seminar on psychological symptoms, physical symptoms and inflammatory biomarkers of IBD^[161]. The BBMW group had significant improvement on Brief Symptom Inventory 18, Beck Anxiety Inventory, Beck Depression Inventory, IBD Questionnaire, and Perceived Stress Questionnaire. Interestingly, median C-reactive protein (CRP) values decreased significantly in the BBMW group but no significant change in CRP values were seen in the educational seminar group^[161] (LOE 1b).

Risk of relapse: Low stress was associated with reduced relapse in a cohort study of 101 patients with quiescent CD followed for one year^[159]. The INSPIRE study evaluated the role of stress management psychotherapy in active IBD patients with high scores (> 60) on the perceived stress questionnaire^[161]. One hundred and fourteen patients were divided into two groups, the first group received usual treatment and the second group received usual treatment and psychotherapy. The intervention did not improve disease or reduce relapse; however, there was a small increase in the IBDQ score ($P = 0.009$, mean differences 16.3 ± 6.1 in patients with UC^[161]). Similarly a Cochrane review of 21 studies and 1745 patients did not show any improvement in IBD relapse or remission rates with psychological interventions aimed at reducing stress^[162] (LOE 1b).

Sleep

Sleep disturbances have been strongly associated with IBD and other chronic inflammatory diseases such as Rheumatoid arthritis and Lupus^[163]. Sleep disturbances in these diseases are due to cytokines produced by chronic inflammation that are also known to affect sleep. Inflammation plays a role in regulating sleep and the interplay of inflammatory cytokines and the sleep cycle is complex. In animal studies, increased levels of Interleukin-1 (IL-1) and TNF- α were associated with an increase in NREM sleep^[164,165]. IL-1 at low levels induces NREM sleep and at higher levels, can cause

NREM suppression and sleep fragmentation^[166,167]. Interleukin-6 (IL-6) mediates the acute-phase response. IL-6 has been shown to suppress REM sleep and promote wakefulness in patients with IBD^[168,169]. Nocturnal diarrhea also disturbs sleep in IBD patients; therefore the high prevalence of sleeping disorders in IBD patients is not surprising (LOE 5).

Risk of disease: It has been hypothesized that sleep disturbances are not merely an outcome, but rather a cause of chronic inflammatory diseases, and there is some evidence to support this hypothesis. Sleep is divided into two parts, REM and Non REM sleep. NREM sleep accounts for 80% of total sleep time and is broken down into 4 stages. Stages 3 and 4 of NREM sleep are often referred to as slow-wave sleep (SWS) and are considered the most restorative stages of sleep where the greatest impact from immune regulation occurs. The effects of SWS can lead to a decrease in colon contractility, which is considered the "rest period" for the colon, so alterations in this stage of sleep can have direct effects on GI physiology such as diminished mucosal integrity^[170,171]. When sleep is disturbed in healthy young volunteers, Interleukin (IL)-1 (beta), TNF- (alpha), and IL-6, the 3 major proinflammatory cytokines that are important in IBD are increased^[170,172].

Risk of disease progression: Sleep deprivation was shown to worsen inflammation and delay healing in a murine model of colitis^[173]. IBD patients have poor sleep quality, prolonged sleep latency, and increase use of sleeping pills when compared with healthy controls^[174,175]. Patients with clinically active IBD have significantly worse sleep than patients with inactive disease^[174,176,177] (LOE 2b, 3,5).

Risk of relapse: A cohort study of 3173 subjects showed that poor sleep increases the risk of relapse in patients with inactive CD but not UC^[178]. In another cohort study, IBD patients in clinical remission but with abnormal sleep were at increased risk of relapse at six months when compared to patients in clinical remission with good sleep^[174]. Currently, the evidence is not strong enough to mandate treating sleep disorders in patients with IBD solely for the purpose of improving IBD outcomes, nevertheless, it is worth noting that sleep disorders can be a significant quality of life issue and all patients with IBD should be screened. In the future screening and treatment of sleep disorders might have therapeutic implications in the treatment of IBD (LOE 2b, 2b).

Vaccinations

The effect of vaccinations on the incidence of IBD is controversial. It was previously thought that vaccinations decreased early childhood infections which in

turn may favor the onset of immunologic diseases^[179]. Viral or bacterial components and chemical adjuvants (e.g., Aluminum) contained in many vaccines were also considered risk factors for incident IBD because of the potential risk of stimulating the immune system leading to a deregulated inflammatory response^[180] (LOE 3b, 5).

Risk of disease: It was first reported in 1995 that the measles vaccine increased the risk of developing IBD. Subsequent studies have not shown any association between measles vaccination and IBD. Epidemiologic studies that investigated other vaccines such as BCG, diphtheria, tetanus, poliomyelitis, smallpox, pertussis, rubella, and mumps, have reported conflicting results^[181-183]. A large meta-analysis that included 11 studies (2400 IBD patients and 34000 controls) did not find any significant increased risk of developing IBD after childhood immunization with BCG, diphtheria, tetanus, smallpox, pertussis, measles, mumps, and rubella-containing vaccines^[183]. Interestingly, there was an increased risk of IBD after poliomyelitis vaccination, however the studies included in the meta-analysis had significant heterogeneity amongst them, which limits the generalizability of the results^[183] (LOE 3b, 3a).

Breast feeding

IBD incidence peaks in early adulthood and therefore early environmental exposures are likely to have a profound effect on future IBD susceptibility. Breastfeeding is an early environmental exposure that affects the development of the immune system and the gut microbiome^[184]. Breastfeeding plays a very important role in protecting against early enteric infections^[185]. Human milk contains (1) lactoferrin that prevents the multiplication of bacteria by chelating iron; (2) IgA that prevents the binding of bacteria to the epithelium and also neutralizes toxins; and (3) Lactadherin that prevents the binding of rotavirus; the latter is the leading cause of gastroenteritis in infants. Other components of human milk that are protective against infections are lysozyme, MUC1, C3, defensins and fibronectin^[186]. The gut microbiome is regulated by breastfeeding as it inhibits the growth of some bacteria due to its anti-bacterial components and promotes the growth of certain bacteria such as *Bifidobacterium* and *Lactobacillus* by producing growth factors^[184]. Breast milk has anti-inflammatory properties, lactoferrin binds to bacterial toxins such as LPS, PAF-acetyl hydrolase and breaks down inflammatory mediators, IL-10, and TGF-1 modulate inflammatory leucocytes^[186]. A meta-analysis of case-control studies with significant heterogeneity found that breastfeeding was protective for both CD and UC^[187]. However, other studies have shown that breastfeeding is either a risk factor or has no association with IBD^[188,189]. Further research is needed to clarify the direction of the association

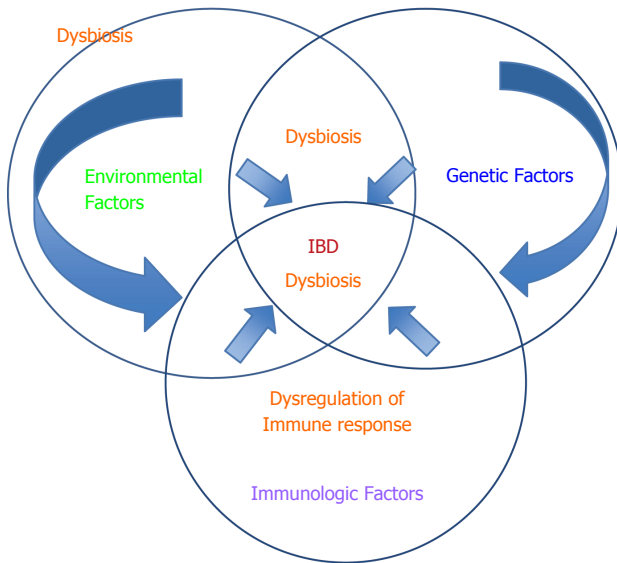


Figure 1 Schematic representation of risk factors contributing to the development of inflammatory bowel diseases. IBD: Inflammatory bowel diseases.

between breastfeeding and IBD (LOE 2b, 3a, 3b).

Appendectomy

Risk for Crohn's disease: The association between appendectomy and CD is conflicting and there has been no consistent association between both entities. Some studies have shown that appendectomy is a risk factor for the development of CD^[190,191]. In contrast, other studies have shown no association with CD^[192]. A meta-analysis showed an increased risk of CD following an appendectomy, however the risk decreased to baseline after 5 years^[193] (LOE 3a, 3b).

Risk for ulcerative colitis: Murine models have suggested that removal of the appendix could have an immune modulating effect that protects against UC^[194]. Observational studies in humans have shown that appendectomy is inversely related to UC^[195,196]. The exact mechanism by which appendectomy protects against UC is unclear. A case-control study showed that patients who had an appendectomy had a significantly lower incidence of UC than matched controls^[195]. It is interesting to note that this held true only for patients who had an appendectomy done for appendicitis or mesenteric lymphadenitis^[195]. The relationship did not hold true for appendectomies done for non-specific abdominal pain (*i.e.*, appendix is found to be normal on post-operative pathology). Moreover, the inverse relationship of appendectomy to the risk of UC was only seen when the surgeries were done prior to the age of 20 years^[195] (LOE 2b, 2b, 5).

DISCUSSION

The available evidence suggests that environmental exposures have variable effects on individuals with

IBD. Advances in genetics and immunology have contributed to our understanding of the pathogenesis of IBD. Environmental exposures or exposomes are believed to be the possible missing link to increasing our understanding of the etiology and increased incidence of IBD in recent years. However, the picture is incomplete. Human studies are extremely limited in their ability to test isolated environmental exposures to demonstrate causation or to assess mechanisms of disease. Given the heterogeneity of environmental factors and the fact that none of the other risk factors (genetic predisposition, immune dysregulation, and dysbiosis) can cause IBD on their own; the challenge is how to effectively translate promising results from animal studies to humans, in order to develop models that incorporate the complex interactions between the environment, genetics, gut microbiota, and the immune system (Figure 1). Recent studies on the incidence of IBD in immigrant populations in western countries further support the role of environmental exposures on the incidence of IBD by suggesting that recent immigrants to western countries from countries with a low risk of IBD acquire the risk of IBD in their adopted country rather than their country of origin^[10,26]. However, the impact of modifying specific environmental factors on causation and established disease remain inadequately studied with limited high quality data from interventional studies to guide clinical practice (Table 3 and 4)^[197-210]. Prospective quantification of environmental exposures in patients at risk of IBD (*e.g.*, first degree relatives) at different time points from birth to adulthood may be useful to predict the natural history of IBD. GWAS of SNP-based Gene (G) × Environment (E) analysis using either the case-only test or standard case-control interaction test under different statistical assumptions has been suggested as a means to further study G × G and G × E interactions in IBD^[211,212]. Specifically, the case-only analysis is more statistically powerful and tests the association between the environmental exposure and the SNP of interest in the cases^[213]. Additionally, omics technologies (genomics, transcriptomics, epigenomics and metabolomics) have provided the opportunity to analyze large population-based databases and their associated biobanks to detect metabolites in blood or urine^[213]. Epigenetics is the study of modifications in regulation of gene expression that occur without changes to the DNA sequence at the interface between environment and heritable molecular and cellular phenotypes^[214]. Epigenetic studies have identified MicroRNAs (miRNAs) as regulators of autophagy and intracellular bacterial processing in IBD^[214]. Quantifying epigenetic changes and further sequencing of the gut microbiome to determine if specific “dysbiotic signatures” are consistent with future development of an IBD phenotype could extend our understanding of the etiopathogenesis of IBD with implications for prevention, diagnosis, and treatment. There is a need

Table 3 Effect of environmental factors in Crohn's disease and impact of interventional studies to modify specific environmental factors

Ref.	Disease onset (Incident CD)	Disease progression	Study population and design	Intervention and comparison group	Outcome
Lifestyle					
Smoking ^[11,13] (LOE 2b, 2a)	↑	↑	Cohort study current smokers with CD (<i>n</i> = 474) ^[17] (LOE 2b) Cohort study current smokers with CD (<i>n</i> = 408) ^[18] (LOE 2b)	Smoking cessation counselling Quitters <i>vs</i> non-quitters	Decreased risk of flares, need for surgery and immunosuppressive therapy ^[17] Continuing smokers had more disease relapses, and patients who quit smoking had similar relapse incidence compared with non-smokers ^[18]
Sleep ^[177,178] (LOE 2b)	No data	↑	None	None	No data
Stress ^[158,159] (LOE 2b)	No data	↑?	Adult and adolescent patients with IBD Systematic review of RCTs and quasi-RCTs (<i>n</i> = 1745) ^[162] (LOE 1a)	Multi-modality psychotherapy	No evidence for efficacy of psychological therapy in adult patients with IBD In adolescents, psychological interventions may be beneficial, but the evidence is limited
Diet					
Dietary fat ^[118] (LOE 3a)	n-6 PUFA↑ n-3 PUFA↓	↓	CD in remission Systematic review of RCTs (<i>n</i> = 1039) ^[201] (LOE 1a)	Fish oil n-3 (PUFA) or placebo	Non-significant trend towards lower risk of relapse at 1 yr in fish oil group compared with placebo
Dietary protein ^[118,120] (LOE 3a, 2b)	Animal protein(meat and fish)↑ Vegetable and dairy↓	↔?	Mild- moderate CD RCT (<i>n</i> = 18) ^[197] (LOE 2b)	Restricted diet (red meat + spelt bread) or control diet (low-fiber, low-fat, and high-carbohydrate)	Radiologic and endoscopic improvement in restricted diet group (interpret with caution; small study with limited generalizability)
Dietary fiber ^[118,120] (LOE 3a, 2b)	Fruit and vegetable fiber↓	↓	Inactive or mildly active CD, RCT (<i>n</i> = 352) ^[207] (LOE 1b)	High fiber diet <i>vs</i> low fiber	No difference in disease activity, surgery or hospitalizations
Food additives [Microparticles (MP) ^[130,131] (LOE 5)]	High MP-diet ↑	High-MP diet↑	Active CD RCT (<i>n</i> = 20) ^[203] (LOE 1b) RCT(<i>n</i> = 83) ^[202] (LOE 1b)	Low -MP-diet <i>vs</i> control diet	Decrease in CDAI in smaller trial ^[203] No difference in larger trial ^[202]
Fruits and vegetables ^[118] (LOE 3a)	↓	↓?	CD in remission RCT (<i>n</i> = 22) ^[198] (LOE 1b)	Semi-vegetarian diet or omnivorous diet	Maintenance of remission rates higher on semi-vegetarian diet compared to omnivorous diet
Food antigens ^[128] (LOE 4)	No data	↑	Active and inactive CD RCT (<i>n</i> = 40) ^[129] (LOE 2b) Active CD Systematic review RCTs (<i>n</i> = 334) ^[210] (LOE 1a)	Elimination diet based on IgG positivity to cheese and yeast or sham diet Elemental <i>vs</i> non-elemental diet	Daily stool frequency significantly decreased by 11% during a specific diet compared with a sham diet. Abdominal pain reduced and general well-being improved ^[129] No difference in the efficacy between elemental and non-elemental diet ^[210]
Enteral nutrition	No data	↔	Active CD Systematic review (<i>n</i> = 192) ^[210] (LOE 1a)	Enteral nutrition <i>vs</i> corticosteroids	Enteral nutrition less effective than corticosteroids for induction of remission
Breastfeeding ^[187-189] (LOE 3a, 3b, 2b)	↔	No data	None	None	No data
Pharmacologic agents					
Nsaids ^[139-140] (LOE2b)	↑?	↑	Inactive IBD with arthralgia. Open label trial (<i>n</i> = 32) ^[144] (LOE 2b)	Rofecoxib 25 mg or 12.5 mg x 20 d	41% responded with reduction in arthralgia scores. <i>P</i> < 0.05. No IBD flares 9% developed GI side effects
Oral contraceptives ^[150,151,153,155] (LOE 3a, 2b)	↔	↔	None	None	No data
Antibiotics ^[145-147] (LOE 3b, 3a)	Early exposure↑	↓	Active CD Systematic review of RCTs (<i>n</i> = 1160) ^[71] (LOE 1a-)	Antibiotic or placebo	Antibiotics superior to placebo at inducing remission
Vaccination ^[183] (LOE 3a)	No effect	No effect	None	None	None
Gut microbiome					
Dysbiosis ^[80-82] (LOE 4)	↑	↑	Mild-moderate CD Systematic review of RCTs (<i>n</i> = 746) ^[199] (LOE 1a)	Probiotics, prebiotics and synbiotics or placebo	Insufficient data to recommend probiotics for use in CD
Ecological (Abiotic)					
Air pollution ^[33,34] (LOE 2c, 3b)	↑	↑?	None	None	No data

Water pollution ^[36-38] (LOE 5)	↑?	↑?	None	None	No data
Low Vitamin D ^[42,44,57,59] (LOE 2b, 3a, 2b)	↑	↑	CD in remission RCT (<i>n</i> = 94) ^[51] (LOE 1a) Mild-moderate CD Cohort study (<i>n</i> = 18) ^[60] (LOE 2b)	Vitamin D3 or placebo No comparison group	Lower relapse rates in patients randomized to vitamin D3 1200 IU/d ^[51] 24 wk of vitamin D3 (up to 5000 IU/d) reduced mean CDAI scores by 112 ± 81 points from 230 ± 74 to 118 ± 66 (<i>P</i> < 0.0001). Quality-of-life scores also improved following vitamin D supplementation ^[60]
Surgery Appendectomy ^[192,193] (LOE 3b, 3a)	↔	No data	None	None	No data

↑ : Increased risk; ↔ : Equivocal risk; ↓ : Decreased risk; ? : Questionable risk. LoE: Levels of Evidence; CD: Crohn's disease; IBD: Inflammatory bowel diseases; PUFA: Polyunsaturated fatty acid.

Table 4 Effect of environmental factors on ulcerative colitis and impact of interventional studies to modify specific environmental factors

Ref.	Disease onset (incident UC)	Disease Activity	Study population and design	Intervention and comparison group	Outcome
Lifestyle					
Smoking ^[11,20,24] (LOE 2b, 3b, 2a)	Current smoking ↓ Smoking cessation ↑	↓	Mild-moderate UC Systematic review (<i>n</i> = 233) ^[205] (LOE 1a) (<i>n</i> = 81) ^[205] (LOE 1a)	Nicotine or placebo Nicotine or corticosteroids	No evidence for efficacy for nicotine preparations in inducing remission in UC
Sleep ^[176,179] (LOE 2b)	No data	↑	None	None	No data
Stress ^[158,159] (LOE 5, 2b)	No data	↑?	Adult and adolescent patients with IBD Systematic review of RCTs and quasi-RCTs (<i>n</i> = 1745) ^[162] (LOE 1a)	Multi-modality psychotherapy	No evidence for efficacy of psychological therapy in adult patients with IBD In adolescents, psychological interventions may be beneficial, but the evidence is limited
Diet					
Dietary fat ^[118] (LOE 3a)	n-3 PUFA ↓ n-6 PUFA ↑	n-3 PUFA ↓	UC in remission Systematic review of RCTs (<i>n</i> = 148) ^[206] (LOE 1a)	fish oil (n-3 PUFA) or placebo	No difference in risk of relapse between n-3 PUFA compared with placebo
Dietary milk ^[116,117] (LOE 5)	↑	No data	Active UC RCT (<i>n</i> = 77) ^[209] (LOE 2b)	Milk-free diet or sham diet	Fewer relapses on milk-free diet than on sham diet
Dietary protein ^[118] (LOE 3a)	↑	↑	None	None	No data
Dietary fiber ^[118,120] (LOE 2b)	↔	↔	UC in remission Open label RCT (<i>n</i> = 59) ^[200] (LOE 2b)	Germinated barley food stuff (GBF) + conventional therapy or conventional therapy	Prolonged maintenance of remission in GBF group ^[200]
			UC in remission Open label RCT (<i>n</i> = 105) ^[122] (LOE 2b)	Plantago ovata or Mesalamine	Plantago ovata as effective as Mesalamine in maintenance of remission ^[122]
Food antigens ^[128] (LOE 4)	↑?	No data	None	None	No data
Food additives ^[131,132] (LOE 5)	↑?	No data	None	None	No data
Breastfeeding ^[187,189] (LOE 3a, 3b, 2b)	↔	No data	None	None	No data
Medication					
Nsaids ^[139,140] (LOE 2b)	↑?	↑	Quiescent to mild UC and CD with arthralgia Prospective Open label trial (<i>n</i> = 32)	Rofecoxib 25 mg or 12.5 mg × 20 d	41% responded with reduction in arthralgia scores. <i>P</i> < 0.05. No IBD flares 9% developed GI side effects
Oral contraceptives ^[150,151,153,155] (LOE 3a, 2b)	↑	↔	None	None	No data
Antibiotics ^[145,147] (LOE 3b, 3a)	Early exposure ↔	↓	Active UC Systematic review of RCTs (<i>n</i> = 9 studies) ^[71] (LOE 1a)	Antibiotic or placebo	Antibiotics superior to placebo at inducing remission
Vaccination ^[183] (LOE 3a)	No effect	No data	None	None	No data

Gut microbiome					
Dysbiosis ^[80,83,84] (LOE 4)	↑	↑	Mild-moderate UC Systematic review of RCTs (<i>n</i> = 650) ^[199] (LOE 1a) Active UC RCT (<i>n</i> = 70) ^[204] (LOE 1b) Active UC, RCT (<i>n</i> = 100) ^[206] (LOE1b)	Probiotics + conventional treatment or placebo Fecal microbiota transplant (FMT) or Placebo Ciprofloxacin + E.coli Nissle or placebo + E.coli Nissle	Probiotics effective for induction and maintenance of remission in UC and pouchitis ^[199] FMT induced remission in a significantly greater percentage of patients with active UC than placebo (24% vs 5%) ^[204] No benefit in the use of E. coli Nissle as an add-on treatment to conventional therapies for active UC
Ecological (Abiotic)					
Air pollution ^[33,34] (LOE 2c, 3b)	↑	↑	None	None	No data
Water pollution ^[36-38] (LOE 5)	↑	↑	None	None	No data
Low Vitamin D ^[44,57] (LOE 2a, 2b)	↑	↑	Active UC Cohort study (<i>n</i> = 368) ^[59] (LOE 2b)	Vitamin D3 or No treatment	Reduction in health-care utilization in the vitamin D treatment group
Surgery					
Appendectomy ^[195-196] (LOE 2b, 3b)	↓	No data	None	None	No data

↑ : Increased risk; ↔ : Equivocal risk; ↓ : Decreased risk; ? : Questionable risk. LoE: Levels of Evidence; CD: Crohn's disease; IBD: Inflammatory bowel diseases; PUFA: Polyunsaturated fatty acid; UC: Ulcerative colitis.

for high quality interventional studies that assess the impact of modifying environmental exposures on the natural history and patient outcomes in IBD (LOE 2b, 5).

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Effectiveness of exercise in hepatic fat mobilization in non-alcoholic fatty liver disease: Systematic review

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Abstract

AIM: To investigate the efficacy of exercise interventions on hepatic fat mobilization in non-alcoholic fatty liver disease (NAFLD) patients.

METHODS: Ovid-Medline, PubMed, EMBASE and Cochrane database were searched for randomized trials and prospective cohort studies in adults aged ≥ 18 which investigated the effects of at least 8 wk of exercise only or combination with diet on NAFLD from 2010 to 2016. The search terms used to identify articles, in which exercise was clearly described by type, duration, intensity and frequency were: "NASH", "NAFLD", "non-alcoholic steatohepatitis", "non-alcoholic fatty liver disease", "fat", "steatosis", "diet", "exercise", "MR spectroscopy" and "liver biopsy". NAFLD diagnosis, as well as the outcome measures, was confirmed by either hydrogen-magnetic resonance spectroscopy (H-MRS) or biopsy. Trials that included dietary interventions along with exercise were accepted if they met all criteria.

RESULTS: Eight studies met selection criteria (6 with exercise only, 2 with diet and exercise with a total of 433 adult participants). Training interventions ranged between 8 and 48 wk in duration with a prescribed exercise frequency of 3 to 7 d per week, at intensities between 45% and 75% of VO_2 peak. The most commonly used imaging modality was H-MRS and one study utilized biopsy. The effect of intervention on fat mobilization was 30.2% in the exercise only group and 49.8% in diet and exercise group. There was no difference between aerobic and resistance exercise intervention, although only one study compared the

two interventions. The beneficial effects of exercise on intrahepatic triglyceride (IHTG) were seen even in the absence of significant weight loss. Although combining an exercise program with dietary interventions augmented the reduction in IHTG, as well as improved measures of glucose control and/or insulin sensitivity, exercise only significantly decreased hepatic lipid contents.

CONCLUSION: Prescribed exercise in subjects with NAFLD reduces IHTG independent of dietary intervention. Diet and exercise was more effective than exercise alone in reducing IHTG.

Key words: Non-alcoholic fatty liver disease; Exercise; Diet; Fat mobilization; Lifestyle modification

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Core tip: Non-alcoholic fatty liver disease (NAFLD) is among the leading causes of chronic liver disease with an increasing prevalence worldwide. Diet and exercise are the mainstay of therapy for patients with NAFLD. This systematic review revealed that both aerobic and resistance exercise, independent of any other intervention, are successful in increasing hepatic fat mobilization. This effect is augmented by combining exercise with dietary interventions. The findings of this systematic review support that exercise interventions are effective in reducing intra hepatic triglyceride in patients with NAFLD independent of weight loss or dietary manipulation.

Golabi P, Locklear CT, Austin P, Afdhal S, Byrns M, Gerber L, Younossi ZM. Effectiveness of exercise in hepatic fat mobilization in non-alcoholic fatty liver disease: Systematic review. *World J Gastroenterol* 2016; 22(27): 6318-6327 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6318.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6318>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an important cause of chronic liver disease worldwide and represents a spectrum of liver diseases ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH)^[1,2]. Recent studies clearly showed that the global prevalence of NAFLD is approximately 25%^[3,4]. Although NAFLD or simple steatosis is not likely to progress to advanced stages of liver disease, it is associated with cardiovascular disease^[5]. In contrast, NASH may progress to hepatic fibrosis, cirrhosis and hepatocellular carcinoma^[6-8]. The histopathology of NAFLD is characterized by accumulation of liver fat, which exceeds 5% of liver weight in the absence

of excessive amount of alcohol consumption, viral infection or other hepatic etiology. NAFLD is strongly associated with obesity, insulin resistance, and dyslipidemia and is known as the hepatic manifestation of metabolic syndrome^[1,2,9,10].

Lifestyle modification is currently accepted as the first line of treatment for the management of NAFLD and weight loss is the only confirmed effective therapy for the treatment of NAFLD^[11]. Lifestyle modification is a general term whose components often differ. It may be non-specific and is a clinical recommendation rather than a prescription. When health care providers suggest that patients follow recommendations for lifestyle changes, additional specificity is important to provide. The components of lifestyle changes usually include diet and exercise, as well as recommendations about smoking cessation, moderate use of alcohol, attention to sleep and stress reduction^[11,12]. A good example of such recommendations is the possible beneficial effect of a Mediterranean diet. Previous studies pointed out that Mediterranean diet is associated with lower incidence of cardiovascular disease and metabolic disorders^[13].

Exercise is different from activity. Activity refers to any movement requiring energy, that is, not resting. In fact, exercise is not synonymous with physical activity; it is a subcategory of it, a planned, structured, repetitive and purposive subcategory with a specific intensity, frequency and duration^[14]. For most health outcomes, additional benefits occur as the amount of physical activity increases through higher intensity, greater frequency, and/or longer duration. Exercise has been documented to be an effective intervention for reducing intrahepatic fat by reducing hepatic lipogenesis^[15]. In fact, three types of exercise have been reported to be effective. One type is walking and jogging, which are examples of aerobic exercise. This type of exercise is "any activity that uses large muscle groups, can be maintained continuously and is rhythmic in nature"^[16]. The second type of exercise is muscle strengthening, this requires muscles to do a greater amount of work than usual. This is muscle overload and utilizes anaerobic metabolism. Muscle strengthening, also known as resistance exercise increases strength, tone, muscle mass, and/or muscle endurance. Flexibility exercise is the activity such as stretching, designed to increase joint range of motion and extensibility of muscle^[17,18]. The American Gastroenterological Association, the American Association for the Study of Liver Diseases and American College of Gastroenterology, all recommend aerobic exercise as a treatment for NAFLD^[19].

The aim of this study was to conduct a systematic review of the pooled data from adult human trials to investigate the efficacy of exercise (aerobic, resistance or combined) interventions with or without dietary interventions on fat mobilization from liver in patients with NAFLD.

MATERIALS AND METHODS

Data sources and searches

Ovid MEDLINE, PubMed, EMBASE and Cochrane database were searched from 2010 to 2016. Two of the authors (PG and MB) performed literature search. The last search of all databases was done on February 26, 2016. In case of a disagreement of eligibility of a study, the authors discussed the issue with a third author. The database searches were performed using the keywords: ("NAFLD", "non-alcoholic fatty liver disease", "NASH" "non-alcoholic steatohepatitis", "fat", "fatty liver", "steatosis") and ("exercise", "aerobic training", "resistance training", "diet") and ("fat mobilization", "intrahepatic lipids", "intrahepatic triglyceride", "MRI", "MR spectroscopy", "H-MRS", "liver biopsy").

Study selection

The search terms listed above were used to identify articles for consideration. Studies examining the association between exercise and fat mobilization, with duration of at least 8 wk, with participants older than 18 years of age, of any sex or ethnic origin with NAFLD/NASH and diagnosed on the basis of radiological/histological evidence of fatty liver were included. Furthermore, studies that clearly prescribed their intervention by type, duration, intensity and frequency, and provided adherence to study protocol were eligible for inclusion. Randomized trials were included. Trials that included dietary interventions were accepted if they met all criteria. Studies were excluded if they didn't specify specific exercise prescriptions, outcome measures demonstrating an exercise effect (*i.e.*, measures of fitness and/or strength), and quantitative measures of intrahepatic fat. Also, studies or study arms for which dietary supplements, herbal preparations, nutraceutical were the intervention to the study were not included.

Titles and abstracts of studies retrieved were evaluated against eligibility criteria. Each manuscript was assessed for pertinence to the issue of prescribed exercise, quantitative measurement of fat in patients with NAFLD. Studies appearing eligible based on their abstract were read in full. Reference lists from all identified studies were searched for relevant studies. The material used was written in English (Figure 1).

Calculations of change with respect to per cent liver fat were performed on all studies selected. The mean fat reduction from all studies was calculated by determining the mean reduction in fat reported for each study, determining the number of subjects in each study and what per cent of the total patient group from all studies it constituted and totaled the findings. In this fashion we compared per cent fat reduction in the group receiving exercise and those receiving diet plus exercise.

Outcome measures

The primary outcome assessed was a decrease in IHTG as determined by histology or H-MRS.

Liver Biopsy: For the definitive diagnosis and grading of NAFLD, histological examination by liver biopsy is still the gold standard. However, it is being used less frequently and has some well-known limitations, such as the risk of complications, potential sampling errors and variability of pathologic interpretation^[6]. Also, a typical liver biopsy samples only 1/50000 of all liver tissue.

Proton magnetic resonance spectroscopy: Previous studies validated the use of H-MRS for assessing intrahepatic lipid content^[20-23]. The assessment of IHTG by H-MRS is highly reliable as this technique samples a much larger liver volume than can be obtained through routine liver biopsy, minimizing the likelihood of sampling error^[21,24]. Indeed, H-MRS is the most direct MR based method to separate the liver signal into its water and fat components and calculate a signal fraction^[25].

Exercise was classified according to the American College of Sports Medicine guidelines that define exercise intensity according to the maximum oxygen consumption (VO_{2max}) that is reached during exercise and categorize intensity into 5 groups as follows: very light ($< 37\% VO_{2max}$), light ($37\%-45\% VO_{2max}$), moderate ($46\%-64\% VO_{2max}$), vigorous ($64\%-91\% VO_{2max}$) and near maximal to maximal ($> 91\% VO_{2max}$). Exercise duration is divided into two groups; high duration includes exercising daily and at least for 60 min, whereas low duration includes exercising below this threshold^[16,26]. Resistance exercise was defined in terms of number of repetitions per amount of weight lifted. Mean drop in IHTG was calculated after normalizing the contribution of fat reduction in each study. For each study, the ratio of controls to those receiving intervention and the total sample was calculated. This percent of the total was multiplied by the percent fat reduction (or increase) for each study.

RESULTS

There were 364 studies of patients NAFLD for whom exercise was prescribed and there were required outcome measures. An additional 7 studies were identified from review of references. After screening for studies published from 2010-2016, 183 studies were identified. Priority was given to well-powered randomized trials. One hundred and eleven were excluded after review of the abstract because they did not include people with NAFLD, or they did not assess the association between NAFLD, exercise and intrahepatic fat mobilization. Only eight studies met all criteria and were included in this review.

All of these studies were randomized trials. One study compared the efficacy of aerobic and resistance exercise in patients with NAFLD. One study utilized biopsy to measure the effects on hepatic histology. The most commonly employed imaging modality to determine change in hepatic steatosis was H-MRS.

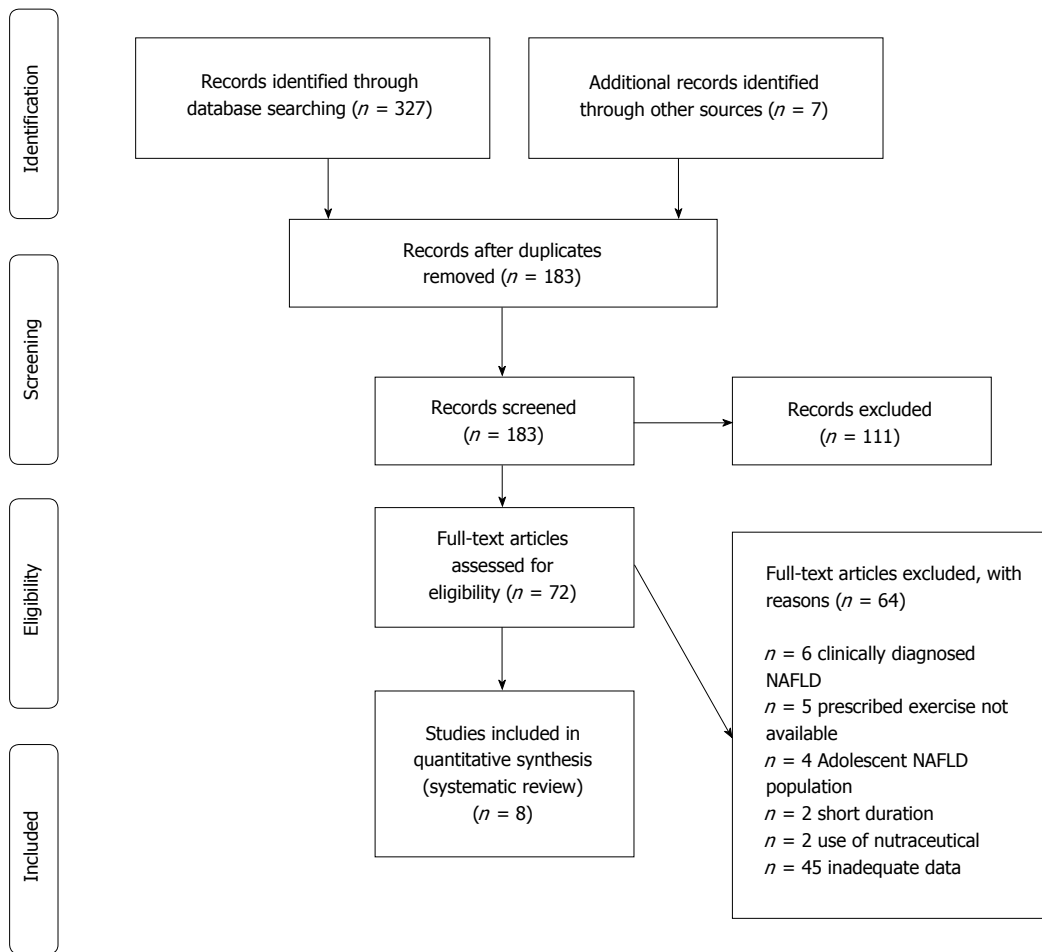


Figure 1 Flow diagram of study.

This analysis combined 8 studies involving a total of 433 adult participants, of which all were randomized trials. In all studies, either aerobic or resistance exercise was prescribed for participants and in only two of them were there dietary intervention. Exercise prescription in studies varied in session duration, intensity, volume (exercise dose) and modality. Dropout rates ranged between 6%-45%. In our analysis, training interventions ranged between 8 and 48 wk in duration with a prescribed exercise frequency of 3 to 7 d per week, at intensities between 45% and 75% of VO_2 peak. Adherence to exercise was monitored using objective measures such as heart rate monitor, blood pressure measurements, pedometer and accelerometers (Table 1).

A total of 350 patients completed the studies. There were 184 included in the exercise only studies (64 controls and 120 received interventions). There were 177 who were treated with diet and exercise (82 controls and 95 received interventions). In the studies that assessed the effect of exercise only, mean drop in %fat was 30.2% in the intervention group and 5.6% in the control group; whereas, in studies with diet and exercise, mean %fat drop was 49.8% for the intervention group and 15.8% in the control group

(Tables 2 and 3).

Exercise only interventions

In their study among 21 patients with NAFLD, Hallsworth *et al.*^[27] analyzed the effects of resistance exercise on IHTG content in the absence of weight loss. In this study, participants performed a moderate intensity/low duration exercise. The intervention group exercised for 3 sessions per week (45-60 min each) for 8 wk. It was found that independent of any change in body weight, resistance exercise reduced IHTG in 8 wk quantified by H-MRS. This study revealed that although no significant changes in blood lipids or ALT were observed, glycemic control, insulin resistance and HOMA scores were improved and there was a 13% relative reduction in hepatic fat in exercise group.

In another study by Sullivan *et al.*^[28] the effects of aerobic exercise on IHTG content were assessed. The participants in the exercise group were prescribed a program of 5 sessions per week (30-60 min each) for 16 wk. This study found that without any change in body weight or % body fat, aerobic exercise had a small but beneficial effect on IHTG. It was reported that a $10.3\% \pm 4.6\%$ relative decrease in IHTG content was seen in the exercise group and a 12.8%

Table 1 General characteristic of studies included

Ref.	Number of subjects with NAFLD	Age (mean)	Sex (male, %)	BMI (mean)	Primary measure	Exercise intervention	Dietary intervention	Program length	Session frequency (wk)	Exercise session duration (min)
Hallsworth <i>et al</i> ^[27] , 2011	21	AE: 52 C: 62	NR	AE: 32.3 C: 32.3	H-MRS	RE	No	8 wk	3	45-60
Sullivan <i>et al</i> ^[28] , 2012	33	E: 49 C: 48	AE: 33 C: 17	AE: 37.1 C: 40	H-MRS	AE	No	16 wk	5	30-60
Bacchi <i>et al</i> ^[29] , 2013	40	AE: 56 RE: 56	AE: 71 RE: 71	AE: 30.5 RE: 28.8	H-MRS	AE and RE	No	4 mo	3	60
Eckard <i>et al</i> ^[33] , 2013	56	LFDE: 44 MFDE: 55 ME: 52 C: 51	LFDE: 50 MFDE: 67 ME: 67 C: 64	LFDE: 32.7 MFDE: 40.3 ME: 31.3 C: 34.7	Liver biopsy	AE	Yes	6 mo	4-7	20-60
Wong <i>et al</i> ^[12] , 2013	154	AE: 51 C: 26	AE: 52 C: 41	AE: 51 C: 25.3	H-MRS, Fibroscan	AE	Yes	12 mo	3-5	30
Pugh <i>et al</i> ^[30] , 2014	31	AE: 48 C: 47	AE: 54 C: 50	AE: 31 C: 30	H-MRS	AE	No	16 wk	3	30-45
Cuthbertson <i>et al</i> ^[31] , 2016	69	AE: 50 C: 52	AE: 77 C: 80	AE: 30.6 C: 29.7	H-MRS	AE	No	16 wk	3-5	30-45
Hallsworth <i>et al</i> ^[32] , 2015	29	AE: 54 C: 52	NR	AE: 31 C: 31	H-MRS	AE	No	12 wk	3	30-40

AE: Aerobic exercise; RE: Resistance exercise; C: Control; LFDE: Low-fat diet plus moderate exercise; MFDE: Moderate-fat diet plus moderate exercise; ME: Moderate exercise; NR: Not reported; RT: Randomized trial; BMI: Body mass index; NAFLD: Non-alcoholic fatty liver disease; H-MRS: Hydrogen-magnetic resonance spectroscopy.

Table 2 Summary of responses in patients receiving exercise only compared to controls

Ref.	Number of subjects with NAFLD	Number of subjects who completed the study	Exercise group	Control group	Percentage of fat reduction in exercise group	Percentage of fat reduction in control group
Hallsworth <i>et al</i> ^[27] , 2011	21	19	11	8	13%	3%
Sullivan <i>et al</i> ^[28] , 2012	33	18	12	6	10%	-8% ³
Bacchi <i>et al</i> ^[29] , 2013 (Aerobic) ¹	40	31	14	-	33%	-
Bacchi <i>et al</i> ^[29] , 2013 (Resistance) ¹	40	31	17	-	26%	-
Eckard <i>et al</i> ^[33] , 2013 ²	56	41	9	11	21%	8%
Pugh <i>et al</i> ^[30] , 2014	31	21	13	8	33%	16%
Cuthbertson <i>et al</i> ^[31] , 2016	69	50	30	20	48%	9%
Hallsworth <i>et al</i> ^[32] , 2015	29	25	14	11	26%	-1% ³

¹Study with 2 different exercise limbs combined; one aerobic, one resistance; ²Study with 4 limbs one of which is exercise; ³Control groups in these studies had an increase in liver fat. NAFLD: Non-alcoholic fatty liver disease.

Table 3 Summary of responses in patients receiving diet and exercise compared to controls

Ref.	Number of subjects with NAFLD	Number of subjects who completed the study	Diet and exercise group	Control group	Percentage of fat reduction in exercise group	Percentage of fat reduction in control group
Eckard <i>et al</i> ^[33] , 2013 ¹	56	41	9	11	27	8
Eckard <i>et al</i> ^[33] , 2013 ¹	56	41	12	11	35	8
Wong <i>et al</i> ^[12] , 2013	154	145	74	71	55	17

¹Study in which 1 limb had low-fat and other moderate-fat diet intake. NAFLD: Non-alcoholic fatty liver disease.

± 3.1% decrease in ALT levels. The authors concluded that as hepatic VLDL-TG secretion rate and VLDL-apoB-100 secretion rate did not exhibit any change, hepatic lipoprotein kinetics remain unchanged.

A randomized trial was conducted in Italy in 2013 to compare aerobic and resistance exercise in type 2 diabetic patients with NAFLD^[29]. In this male dominant

study (22 males vs 9 females), mean ages of both exercise groups were similar (55.6 ± 2 vs 56 ± 2). The aerobic exercise group performed moderate to vigorous intensity exercise for 60 min, 3 sessions per week for 16 wk while resistance exercise group performed 3 series of 10 repetitions at 70%-80% VO_{2max}, with 1 min of recovery between series for

60 min, 3 sessions per week for 16 wk. In H-MRS quantifications at the end of study period, resistance exercise was found to be equally effective as aerobic exercise in reducing hepatic fat mass. Both groups exhibited significant reductions in IHTG content (32.8% in aerobic vs 25.9% in resistance) and showed improvements in HbA1c, HDL, TG levels and insulin sensitivity.

In another randomized controlled trial in United Kingdom, Pugh *et al.*^[30] investigated the associations between hepatic fat and endothelial dysfunction among obese NAFLD patients. Participants in the intervention group performed a supervised aerobic exercise, which was moderate intensity and low duration, and was gradually increased during the study period of 16 wk. It was found that there was a 33.3% change in IHTG by H-MRS in the exercise group and 16.8% in the control group, as well as improvements in the flow mediated dilatation on brachial arteries. They concluded that moderate intensity exercise can improve endothelial dysfunction and reduce the risk of cardiovascular disease.

Another study was conducted among 69 patients with NAFLD to determine if there was dissociation between exercise-induced reduction in liver fat and changes in hepatic and peripheral glucose homeostasis in NAFLD^[31]. In this randomized controlled trial, patients were randomly assigned to either 16 wk of supervised exercise or conventional counselling. Intensity of the exercise started from 3 times a week, for 30 min per session, reaching 30% of heart rate reserve and increased to 5 times a week, for 45 min per session and reaching to 60% of heart rate reserve. After 16 wk, IHTG content significantly decreased from 19.4% to 10.1% in the exercise group, but not in the control group (from 16% to 14.6%). There was a significant difference with the amount of weight reduction between two groups (mean change -2.5 Kg in exercise group and 0.2 in control group). Although liver function tests decreased in both groups, it was not statistically significant. In the exercise group, peripheral insulin resistance improved as opposed to hepatic insulin resistance.

In another randomized controlled study by Hallsworth *et al.*^[32], the effect of high-intensity interval training on liver fat, cardiac function and metabolic control in patients with NAFLD was assessed. Twelve weeks of cycle ergometry three times per week resulted in reduction in H-MRS measured IHTG of 27%. Exercise also resulted in reduction in fat mass (mean 1.8 kg), plasma ALT, AST and improvement in cardiac diastolic function, but with limited impact on glucose control.

Exercise and diet interventions

We identified two eligible articles that studied 177 participants in which both diet and exercise were prescribed^[12,33]. In the study by Wong *et al.*^[12], the

intervention involved a community-based lifestyle modification was for 12 mo, moderate in intensity and low in duration. Seventy-four patients were in the diet and exercise group and 71 patients were in the control. The two groups were well-matched in demographic characteristics, clinical and laboratory data, IHTG, and liver stiffness measurements. In this study 64% of patients achieved remission of NAFLD in the intervention group and 20% in the control group. Patients in the intervention group had greater reduction in body weight (5.6 kg), BMI and waist circumference, total cholesterol, LDL, ALT and liver stiffness. Also, in intervention group IHTG component reduced by 6.6% as compared to 2.1% in control group ($P < 0.001$).

The second study conducted with diet and exercise included 56 biopsy proven NAFLD patients who were divided into four groups as follows: low fat diet and moderate exercise, moderate fat diet and moderate exercise, moderate exercise, and control^[33]. Exercise prescribed was moderate in intensity and low in duration, for a total of 6 mo. The effects of the interventions were evaluated at the end of study with a repeat liver biopsy. It was found that low or moderate fat diet and moderate exercise significantly decreased the mean NAFLD activity scores (NAS) in 6 mo. By comparison, the change in NAS in the exercise only and control groups was not significant. It was also stated that weight loss was not necessary for improvement in liver histology. The data are summarized in Table 4.

DISCUSSION

This systematic review assessed the published literature to determine the efficacy of exercise interventions in modifying the amount of IHTG in adults. The results suggest that regardless of type, exercise reduces the amount of IHTG in patients with NAFLD. In fact, the beneficial effects of exercise on intrahepatic lipids are seen even in the absence of significant weight loss. Although combining exercise program with dietary interventions augments the reduction in IHTG, as well as improves measures of glucose control and/or insulin sensitivity, exercise only can also significantly decreases hepatic lipid contents. Also, it is emphasized in the recent guidelines that for patients with NAFLD, the choice of training should be tailored based on patients' preferences to be maintained in the long term^[34]. It can be suggested that exercise 3-4 times a week, at 20-40 min per session with achieving 70% VO_{2max} is ideal for mobilizing fat from liver among NAFLD patients. This is considered a moderate level.

Exercise is considered to be one of the most effective, non-pharmacological interventions in the treatment of nonalcoholic fatty liver disease^[35,36]. Although the protective effects of exercise on metabolic disease was demonstrated many decades ago, still relatively little is known about the underlying molecular

Table 4 Summary of studies included

Ref.	Intervention	Changes in fat	Physiologic changes	Clinical outcome	Exercise outcome
Hallsworth <i>et al</i> ^[27] , 2011	RE	13% relative decrease in IHTG in exercise group	No significant change in blood lipids or ALT Approximately 12% increase in insulin sensitivity and increased fat oxidation	No effect on body weight, visceral adipose tissue volume or whole body fat	RE without weight change is effective in reducing IHTG in people with NAFLD
Sullivan <i>et al</i> ^[28] , 2012	AE	10.3% ± 4.6% relative decrease in IHTG in exercise group	Plasma ALT decreased 12.8% + 3.1 in exercise group	Body weight, body fat mass remained same	Small decrease in IHTG content
Bacchi <i>et al</i> ^[29] , 2013	AE and RE	Reduction in IHTG by 35.8% in AE vs 25.9% in RE	HbA1c, HDL, TG, insulin sensitivity improved	BMI, total body fat mass, VAT, SAT were reduced	Absolute and relative reduction in IHTG in both exercise groups
Eckard <i>et al</i> ^[33] , 2013	Diet and AE	Significant change was found in pre to post NAFLD activity score	Significant decrease in Brunt grade, ALT, AST	No subgroup achieved a significant weight loss of > 5% Changes in % body fat were minimal	Lifestyle modification improved liver histology after 6 mo intervention Weight loss is not the key to improving liver histology
Wong <i>et al</i> ^[12] , 2013	Diet and AE	6.7% decrease in IHTG in intervention group	Decrease in Total cholesterol, LDL, ALT and liver stiffness	Reduction in body weight 5.6 kg, in BMI and waist circumference	64% of patients achieved remission of NAFLD in exercise group
Pugh <i>et al</i> ^[30] , 2014	AE	IHTG decreased by 33% in exercise group SAT decreased no significant difference in VAT, total abdominal fat and muscle fat	Fasting glucose decreased No difference in HOMA score, insulin, liver enzymes, lipid profile, adiponectin, and leptin	No weight change Cardiorespiratory fitness improved Waist circumference decreased	improved endothelial dysfunction in the absence of change in liver fat and visceral fat content exercise training can reduce intrinsic CVD risk in NAFLD
Cuthbertson <i>et al</i> ^[31] , 2016	AE	IHTG Significantly decreased (19.4%→10.1% in AE, 16%→14.6% in control)	No significant change in HOMA, plasma insulin, fetuin, irisin, adiponectin	Cardiorespiratory fitness improved in exercise group	Improvement in peripheral IR but not in hepatic IR
Hallsworth <i>et al</i> ^[32] , 2015	AE	27% reduction in IHTG in exercise group	Decrease in ALT and AST Improvement in diastolic function	No weight change Mean 1.8 kg reduction in fat mass and body fat percentage	Significant reduction in IHTG, liver enzymes and body fat

H-MRS: Proton magnetic resonance spectroscopy; RE: resistance exercise; AE: Aerobic exercise; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IR: Insulin resistance; HOMA: Homeostasis model assessment of insulin resistance; HbA1c: Glycosylated hemoglobin; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglyceride; BMI: Body mass index; IHTG: Intrahepatic triglyceride.

mechanisms. The most striking hepatic adaptation to exercise is the decrease in hepatic lipid content, even when overall weight loss is not observed^[37,38]. Our main findings are in agreement with previous systematic reviews and meta-analyses^[39-41]. Keating *et al*^[39], aimed to assess the efficacy of aerobic or resistance training on both hepatic fat and ALT levels and demonstrated that exercise alone was effective on fat mobilization from liver. On the other hand, the authors concluded that there was no significant difference in either ALT levels or total body weight between the exercise and control groups^[39]. Another systematic review aimed to assess the efficacy of lifestyle interventions and included diet only interventions along with exercise only interventions and combined studies. It was noted that weight reductions of 4%-14% resulted in significant reductions in IHTG levels of 35%-81%. It was also stated that exercise could lead to decrements in IHTG and weight loss was not a prerequisite for this change^[40].

In contrast to the findings of this review, in a study that did not meet our inclusion criteria (study population was not restricted to subjects with NAFLD),

the investigators found that calorie restriction only was equal to calorie restriction with exercise in reducing liver fat^[42]. The authors stated that there was no additive effect of exercise training. The two major caveats of this study were: CT was used to assess IHTG and some participants at the baseline did not have intrahepatic fat accumulation.

One of the limiting factors of this study is that there are relatively few studies that meet all criteria for inclusion and that many of the studies had a small number of participants (< 100 subjects). This is not uncommon for exercise intervention studies that require behavioral change to assure adherence for a relatively long period, because one often needs > 8 wk to see an increase in aerobic capacity or strength. However, the authors arbitrarily used very strict criteria for what was considered exercise and which outcomes would be acceptable for determining an exercise effect. The former required that studies list the frequency and intensity not only the term "exercise" or "activity", to qualify. The outcomes needed to include standard physiological assessments of exercise such as heart rate, or oxygen consumption. This assures that the

exercise is actually performed. Secondly, it is often difficult to keep patients motivated to participate in this type of interventional study. We believe that the relative infrequency of studies performed for exercise-induced intrahepatic fat reduction is, in part due to these factors.

One of the most significant challenges we face in utilizing this effective treatment is adherence to exercise. Interpretation of the cumulative data from these reviews suggests that one strategy to increase adherence might be to target exercise and not substantially limit dietary intake or change the ratios of macronutrients. Additionally, selecting an exercise program that targets 50%-70% of heart rate maximum is likely to be well tolerated and not to be experienced as too challenging. In other words, this level of intensity is not likely to require the exercise to be done in anaerobic range, thereby minimizing discomfort.

In conclusion, this systematic review permits a pooling of studies that met strict criteria for measures of intrahepatic fat and a prescribed exercise intervention. An exercise intervention of moderate intensity is effective for the mobilization of IHTG. The findings support the view that exercise is effective in reducing IHTG in patients with NAFLD independent of weight loss or dietary manipulation. Combining exercise with dietary interventions augments the reduction in IHTG.

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COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) has become a significant healthcare problem around the world. Lifestyle modifications are the cornerstone of treatment for the management of NAFLD. NAFLD can be reversed by reducing intrahepatic fat content which may decrease the undesired hepatic and metabolic effects. This is why the authors recommend for regular exercise healthy eating plan for these patients.

Research frontiers

Multiple studies have tried to assess the effect of exercise for hepatic fat mobilization with or without combination dietary interventions. Studies have focused on both aerobic and resistance type of exercise, based on the duration, intensity and frequency of exercise. Most of the studies used moderate intensity/low duration exercise as an intervention. The details of dietary interventions include the amount of carbohydrates and fat in diet.

Innovations and breakthroughs

Both aerobic and resistance exercise interventions have been shown to be effective in reducing intrahepatic triglyceride content. The addition of dietary interventions augments hepatic fat reduction of exercise. Exercise interventions are successful in mobilizing fat from liver tissue, independent of weight loss.

Applications

The studies selected for this systematic review support the benefits of lifestyle modifications. An exercise intervention of moderate intensity is effective for

the mobilization of intrahepatic triglycerides. Combining exercise with dietary intervention augments the success of lifestyle modification for hepatic fat reduction.

Terminology

In most studies, in order to assess the change in intrahepatic fat content, hydrogen-magnetic resonance spectroscopy (H-MRS) is utilized. H-MRS is a non-invasive magnetic resonance imaging based imaging technique which is highly reliable for assessing hepatic parenchyma. It works by separating liver signal into water and fat components from which one is able to calculate a signal fraction.

Peer-review

In this systematic review, the authors aimed to evaluate the effect of exercise on hepatic fat content via conducting a broad literature search with strict inclusion criteria. The approach was performed in a careful, systematic way in order to determine the level of evidence for exercise as an effective mode for mobilizing fat from the liver.

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Bile cast nephropathy: A case report and review of the literature

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Abstract

Bile cast nephropathy is a condition of renal dysfunction in the setting of hyperbilirubinemia. There are very few cases of this condition reported in the last decade and a lack of established treatment guidelines. While the exact etiology remains unknown, bile cast nephropathy is presumed to be secondary to multiple concurrent insults to the kidney including direct toxicity from bile acids, obstructive physiology from bile casts, and systemic hypoperfusion from vasodilation. Therapy directed at bilirubin reduction may improve renal function, but will likely need dialysis or plasmapheresis as well. We report our case of bile cast nephropathy and the therapeutic measures undertaken in a middle-aged male with chronic renal insufficiency that developed hyperbilirubinemia and drug-induced liver injury secondary to antibiotic use. He developed acute renal injury in the setting of rising bilirubin. He subsequently had a progressive decline in renal and hepatic function, requiring dialysis and plasmapheresis with some improvement, ultimately requiring transplantation.

Key words: Bile cast; Dialysis; Drug-induced liver injury; Plasmapheresis; Hyperbilirubinemia

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Core tip: The role of bilirubin in causing acute renal insufficiency is not well known. Our case report is one of few documenting evidence of renal insufficiency as a result of hyperbilirubinemia. Diagnosis requires a high index of suspicion in patients with hyperbilirubinemia

with concomitant acute renal insufficiency. Renal biopsy is the solitary means of definitive diagnosis. Treatment is targeted at improving hepatic dysfunction and decreasing bilirubin burden. Numerous treatment modalities to reduce bilirubin have been suggested with variable outcomes.

Patel J, Walayat S, Kalva N, Palmer-Hill S, Dhillon S. Bile cast nephropathy: A case report and review of the literature. *World J Gastroenterol* 2016; 22(27): 6328-6334 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i27/6328.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i27.6328>

INTRODUCTION

Cholestatic liver disease has a number of consequences on renal function. Most of the clinical manifestations are secondary to hemodynamic changes resulting in a pre-renal state. In patients with profound hyperbilirubinemia, bile casts result in direct toxicity to the nephron. It is commonly known as bile cast nephropathy and has been described under various terms including cholemic nephrosis, biliary nephrosis and jaundice-related nephropathy. This entity has been reported as early as 1899 when patients with jaundice and renal failure were found to have bile cast deposition in their kidneys on biopsies. Only a handful of cases were reported until van Slambrouck *et al*^[1] published a study of 44 patients with bile cast nephropathy. It is characterized by the presence of bile casts on renal biopsy in the setting of hyperbilirubinemia and renal insufficiency^[1,2]. Its prevalence is likely greater than previously recognized. Early recognition and treatment are essential as they may lead to reversal of symptoms and improved prognosis. Presently, there is little literature about the disease and its etiology with only a few case reports in the past decade^[3-8].

Herein, we report a case of bile cast nephropathy in a patient who developed acute renal injury in the setting of rising bilirubin and with improvement as bilirubin levels decreased.

CASE REPORT

A 54-year-old Caucasian male presented to the emergency department with a 3 wk history of progressive jaundice, intractable pruritus, and anorexia. Two weeks prior to symptom onset, he was diagnosed with chronic left heel osteomyelitis and was started on antibiotic therapy with Piperacillin/tazobactam. At the time of evaluation, signs and symptoms of hepatic dysfunction were persistent despite discontinuation of antibiotic therapy. His past medical history was significant for well-controlled diabetes mellitus (most recent Hgb A1c of 5.6), hypertension, chronic sinusitis, chronic renal insufficiency (baseline creatinine

of 2.1) and hyperlipidemia. Surgical history included a distant cholecystectomy and a partial nephrectomy for a complex renal cyst. Family history was negative for any liver or kidney disease. Social history was negative for alcohol use or tobacco use, intravenous drug abuse, tattoos, history of blood transfusions or high risk sexual behavior. On physical exam, he was alert and oriented with jaundice, a distended abdomen, and a 1+ bilateral pitting edema of his lower extremities. The rest of the physical exam was unremarkable. Laboratory studies at the time of presentation were significant for a sodium of 135 mmol/L, potassium of 4.4 mmol/L, BUN of 40 mg/dL, creatinine of 2.13 mg/dL, albumin of 2.8 g/dL, AST of 121 U/L, ALT of 129 U/L, alkaline phosphatase of 851 U/L, a GFR of 33 and a total bilirubin of 19.3 mg/dL. His CBC was significant for hemoglobin of 11.5 g/dL, MCV 95.6 fL, RDW 18.2, WBC 10.56/mcl, platelet count 506/mcl and his INR was 1.4. A urinalysis was unremarkable with the exception of trace protein and presence of bile. At baseline, he had normal liver enzyme values and a creatinine of 2.1 mg/dL. An ultrasound of the abdomen revealed mild splenomegaly, ascites, and absence of biliary ductular dilation. A subsequent CT and MRCP were also negative for evidence of biliary pathology. The patient was admitted with a presumptive diagnosis of drug-induced liver injury (DILI) with the differential diagnoses including acute viral hepatitis, autoimmune liver disease, and acute decompensation of chronic liver disease. His workup was negative for HAV, HBV, HCV, EBV, and HSV. Ceruloplasmin levels and autoantibodies were also negative. He was treated supportively with intravenous hydration, initiation of ursodeoxycholic acid, cholestyramine and N-acetylcysteine for possible DILI. The patient experienced continued deterioration of his hepatic and renal function. A trans-jugular liver biopsy revealed portal and peri-portal fibrosis (stage 1-2), marked hepatocanalicular cholestasis with focal bile infarcts and multiple pseudo-ground glass inclusions within the hepatocytes consistent with DILI. Additionally, his peri-hepatic pressures were normal. The patient's renal function continued to progressively deteriorate with increasing azotemia and oliguria. Laboratory data at this time was significant for a urine sodium less than 20 mmol/L, BUN of 81 mg/dL and creatinine of 5.1 mg/dL, suggesting a pre-renal origin with hepatorenal syndrome in the differential diagnosis. His urine analysis revealed 3+ RBCs, 5-10 WBCs, positive leukocyte esterase and presence of eosinophils. The presence of eosinophils suggested an immune-mediated process such as a vasculitis or interstitial nephritis. His ANCA, complement C3, and C4 levels however, were normal.

The patient began empiric therapy with levofloxacin and steroids due to concerns of a urinary tract infection, rapidly progressive glomerulonephritis, and/or interstitial nephritis. Urine cultures were positive for *E. coli* and his levofloxacin was changed to meropenem. During this time, his renal function

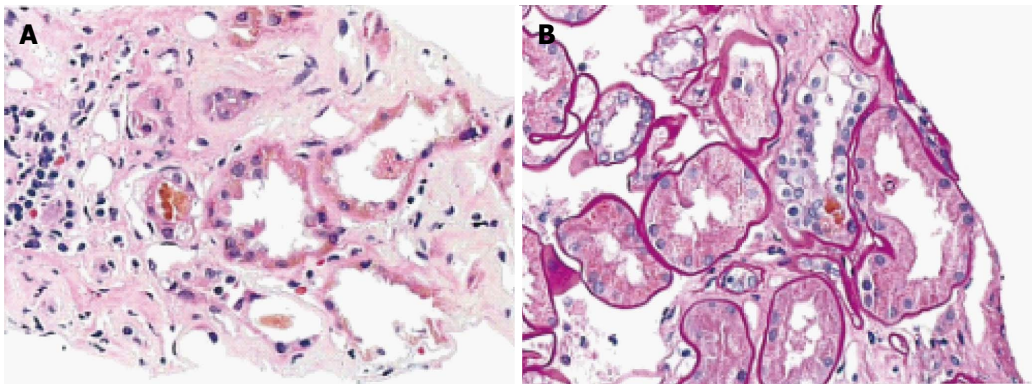


Figure 1 Kidney biopsy demonstrating bile casts and droplets (A and B).

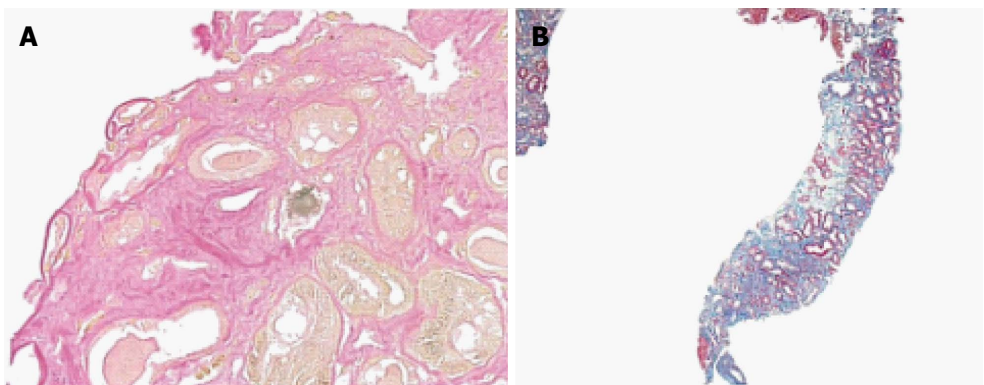


Figure 2 Kidney biopsy with positive Fouchet stain indicating presence of bile (A) and showing tubular atrophy and interstitial fibrosis (B).

Table 1 Creatinine and bilirubin levels over time															
Date	On admission	18-Feb	26-Feb	3-Mar	6-Mar	12-Mar	15-Mar	25-Mar	26-Mar	1-Apr	15-Apr	20-Apr	21-Apr	7-May	9-May
Treatment date	0	28	36	41	44	50	53	63	64	70	84	89	90	106	108
					Dialysis Initiated			Plasmapheresis Initiated				CVC removed			
Creatinine	2.13	2.89	4.18	4.92	5.47	1.75	2.58	3.63	3.49	3.01	3.6	4.26	4.43	5.14	4.73
T. Bili	19.3	18.2	22.9	26.1	29.0	19.5	17.9	25.3	20.4	12.6	10.9		18.0	29.7	27.1

Initiation of dialysis, plasmapheresis (TPE), and central line catheter removal (CVC) are indicated. N/A indicates data not available.

continued to decline and he underwent a renal biopsy that revealed thickening of the glomerular basement membrane and sclerosis of Bowman’s capsule. In addition, both proximal and distal tubules contained pigmented bilirubin casts and droplets (Figure 1). The cross sections stained positive with Fouchet’s stain (Figure 2A). Lastly, tubular atrophy and interstitial fibrosis were seen (Figure 2B). Immunostaining was negative for IgG, IgM, C3, C4, C1q, albumin, as well as kappa and lambda light chains.

Hemodialysis (HD) was initiated while the patient continued treatment with steroids, ursodeoxycholic acid, and cholestyramine. After approximately two weeks of HD, his bilirubin persisted in the 20 mg/dL range and he remained oliguric (Table 1). Subsequently, plasmapheresis and diuretic therapy with furosemide and spironolactone were initiated with a decrease in

bilirubin to 10 mg/dL after multiple treatments and improvement in oliguria. He was closely monitored for any infectious or bleeding complications and was discharged home with continued plasmapheresis as needed to maintain plasma bilirubin levels of approximately 10 mg/dL and under.

He returned to the hospital 2 wk post-discharge with a central venous catheter infection, requiring removal of his temporary dialysis catheter and cessation of plasmapheresis. Despite cessation of plasmapheresis, the patient was maintaining adequate urine output of approximately 1L per day with stable electrolyte and acid-base status. However, he rapidly developed recurrent hyperbilirubinemia (T. bili 29.7 mg/dL) and progressive renal dysfunction (Cr 5.14, GFR 12-13, urine sodium 89 mmol/L, bilirubinuria and bilirubin crystals present in urine). He was also noted to have

Table 2 Most recent available creatinine and total bilirubin values

Date	February 22, 2016
Creatinine (mg/dL)	0.96
T. Bili (mg/dL)	0.5

ascites and required large volume paracentesis with protein gradients consistent with portal hypertension-related ascites. A repeat liver biopsy was performed which showed stage 3-4 bridging fibrosis with focal nodule formation along with prominent bile ductular proliferation and focal ductular cholestasis. In light of his MELD score of 38, the patient underwent a successful liver and kidney transplant with resolution to normal of his hepatic and renal indices (Table 2).

DISCUSSION

Bile cast nephropathy is a rare and poorly understood entity characterized by progressive renal insufficiency in the setting of elevated serum bile salts and hyperbilirubinemia. Elevated total bilirubin levels, typically greater than 20 mg/dL, are reported in cases of bile cast nephropathy. Both direct and indirect bilirubin may be involved, suggesting a primary hepatocellular dysfunction. Renal biopsies in these patients typically show bile cast formation in the setting of elevated bilirubin and serum creatinine levels^[5].

History

This entity was first described by Qunicke in 1899, when autopsies from patients with acute onset jaundice and renal failure showed deposition of bile pigments in the renal glomeruli. In 1922, Hessler showed that severe jaundice was associated with the presence of marked granulated cells and free bilirubin in the urine of dogs and humans, suggesting that the accumulation of bile in the renal cortex was nephrotoxic^[9]. Subsequently, in 1937, Elsom^[10] also observed that jaundice was associated with impairment of renal function that was reversible with the resolution of hyperbilirubinemia. In 2006, Betjes and Bajima used the term "jaundice-related nephropathy" for the historical term cholemic nephropathy for changes ranging from proximal tubular dysfunction to renal failure due to the deposition of bile and bile salts^[4].

Etiology

The exact etiology remains unknown, but any insult leading to profound bilirubinemia (whether hepatic or extrahepatic) may progress to this condition. Alcohol may exacerbate or be an etiologic factor with this entity, as all ten patients in van Slambrouck's study with cirrhosis secondary to alcoholism had bile casts present on autopsy. These casts were absent, however, in all patients with cirrhosis secondary to hepatitis

C (5 patients) or with indirect hyperbilirubinemia (2 patients)^[1]. Other reported etiologies of bile cast nephropathy included patients with hyperbilirubinemia as a result of infectious mononucleosis and steroid use^[3,5]. Yet another case involved a patient with colorectal cancer 3 wk after a wedge resection^[8].

This disease entity likely represents a broad spectrum of disease, from mild reversible changes in those without underlying renal dysfunction and hyperbilirubinemia of short duration, to irreversible progressive disease in those with underlying renal insufficiency with prolonged and severe hyperbilirubinemia. The exact mechanism in which bile and bile salts cause acute tubular injury remains unknown, however, various mechanisms are worth considering.

During cholestasis, hepatocytes attempt to export bile acids to prevent intracellular damage by inducing basolateral bile acid pumps. The kidneys, similarly, undergo changes in the proximal tubule to excrete excess bile. This excess bilirubin is believed to cause oxidative damages of the cell membranes of the tubules and uncoupling of mitochondrial phosphorylation at the cellular level^[11,12]. Furthermore, inhibition of the Na-H, Na-K, Na-Cl pumps by sulfated bile salts in proximal tubules and in the loop of Henle may result in pH changes which may enhance bile cast deposition, tubular toxicity and injury^[2,4,13,14]. It has been hypothesized that there is a limit to bilirubin transport in the proximal tubules after which they become saturated, leading to cast formation and tubular obstruction^[1,3]. These findings are supported by a study that demonstrated a direct correlation between the severity of hepatic dysfunction as measured by ALT, and the severity of renal dysfunction^[15]. The 35 patients studied by Elini Baitakari with obstructive jaundice had no underlying renal or chronic hepatic disease and had a baseline conjugated bilirubin of 10 mg/dL. They found changes consistent with proximal tubular damage in the form of glucosuria, phosphaturia, and microglobulinuria. They also reported decreased serum uric acid and phosphate levels which were inversely proportional to the total and indirect bilirubin levels. The treatment of jaundice resulted in improvement of proximal tubule function^[4,13].

Hemodynamic changes resulting in pre-renal azotemia may also be contributory. Elevated levels of bile salts have been demonstrated to have negative chronotropic and ionotropic effects, resulting in cardiovascular instability and decreased renal perfusion. This is further exacerbated by changes in endovascular reactivity believed to be due to the prevalence of endotoxemia, hypoalbuminemia, and nitric oxide-mediated mechanisms resulting in decreased peripheral vascular resistance and decreased renal blood flow, resulting in ischemia to the kidney. Studies in mice support this two-hit mechanism of renal dysfunction^[2,16]. Aoyagi and Lowenstein observed that mice that were infused with bile acid followed by inducing renal ischemia for 30

min developed renal insufficiency. However, mice that were infused with either bile acid alone or underwent induced renal ischemia alone for 30 min did not develop renal insufficiency, supporting the concept of a two hit phenomenon^[17]. While the dysfunction in this study was reversible with removal of the stressors, it is likely that those with chronic underlying disease or prolonged hyperbilirubinemia may result in irreversible changes.

Renal effects of hyperbilirubinemia

Renal dysfunction is exhibited by elevated creatinine levels, pigmented bile crystals on urinalysis, natriuresis, and B2 microglobulinuria^[18]. Urine osmolality has been reported to fall significantly in rats as early as 24 h after bile duct ligation representing an underlying concentrating defect^[19].

Sitprija *et al.*^[2] studied 15 patients with obstructive jaundice secondary to cholangiocarcinoma with no clinical evidence of hemolysis, renal dysfunction, or cardiac dysfunction at baseline. No changes in renal function were observed in patients with bilirubin levels less than 15 mg/dL. At bilirubin levels greater than 26 mg/dL, there was evidence of decreased free water clearance, creatinine clearance, and mean arterial pressure. At bilirubin levels greater than 40 mg/dL, renal perfusion was also decreased. The two patients described by Betjes and Bejemia with obstructive jaundice also had findings consistent with those of Sitprija *et al.*^[2]. These studies found pronounced GFR loss (mean creatinine 3.2 mg/dL) with natriuresis (mean urine sodium 57 mmol/L) in the presence of hyperbilirubinemia (mean T. bili 30 mg/dL) and low serum albumin (mean 34 g/L) concentration^[4]. Glucosuria, phosphaturia, and microglobulinuria have also been reported^[8].

Pathology and histology

The primary findings in bile cast nephropathy include renal tubular hypertrophy, the presence of pigmented bile casts within the renal tubules and absence of glomerular pathology^[20-22]. Other findings reported include tubular damage with evidence of dilatation of the lumen and cytoplasmic vacuolization, as well as the presence coarse granular brown casts^[3]. Electron microscopy findings include dilated mitochondrial cristae and bile acid accumulation within lysosomes. Bile cast presence was more pronounced in the distal segments of nephrons, but was found in more proximal tubules with increasing severity of hyperbilirubinemia^[5]. In this patient, bile cast deposition was seen in both proximal and distal tubules along with ischemic changes.

Historically, studies in patients with hyperbilirubinemia have shown histologic changes including glomerular congestion, nuclear extrusion with vacuolization, and necrosis in proximal convoluted tubules. Additionally, dilation with lymphocytic collection and necrosis in secondary convoluted tubules, interstitial

edema, and bile cast deposition were also seen^[21].

Van Slambrouck *et al.*^[1] studied the renal biopsies of 44 patients with severe liver dysfunction and bile casts were observed in 24 of these patients. Involvement of the distal nephron was found in mild cases (18 patients) and involvement of the proximal tubule was found in severe cases (6 patients). The mean serum total bilirubin was 26.2 mg/dL, direct bilirubin was 16.3 mg/dL, mean serum creatinine was 2.3 mg/dL, and mean albumin was 31 g/L in their patient population with renal casts. Sixty six percent of the patients in their study with bile casts had histological evidence of acute kidney injury giving the strongest evidence to date that bilirubin and bile salts are directly nephrotoxic.

Treatment

Owing to the rarity of this condition, there are currently no accepted treatment guidelines. Interventions to reduce bilirubin burden, such as relief of biliary obstruction *via* ERCP with stent placement and hemodialysis, have been attempted to improve outcomes. While this appears to be an effective strategy early in the disease course, its efficacy in established disease is uncertain^[4,6,7,23]. Plasmapheresis may additionally be of utility.

Other extracorporeal treatment options aimed at reduction of inflammatory cytokines and reduction of bilirubin have also emerged, including the molecular adsorbents recycling system (MARS), coupled plasma filtration adsorption (CPFA), and plasma filtration adsorption dialysis. These methods of blood and/or plasma filtration have served utility in patients with sepsis, acute liver failure and acute on chronic liver failure *via* the filtration of inflammatory cytokines, bilirubin and bile acids, among other compounds such as amino acids and free fatty acids. MARS has shown improved survival rates in patients with acute on chronic liver failure in the setting of sepsis^[24,25]. CPFA has demonstrated efficacy with improved survival rates in early or middle stage liver failure secondary to viral hepatitis^[26]. These newer extracorporeal treatment options may be utilized to allow liver regeneration after acute injury in an effort to circumvent transplantation or at the least, as a bridge until transplantation can occur^[27,28].

Various medical therapies including the use of steroids, cholestyramine, ursodeoxycholic acid, and lactulose have been shown minimal benefit^[4]. Interestingly, lactulose appeared to have a protective role on the kidney in experimental rats. This may have been a result of decreasing the endotoxemia associated with this condition^[29-31]. In our patient, hemodialysis failed to successfully decrease bilirubin levels. Subsequently, daily plasmapheresis was initiated with effective reduction of bilirubin. However, cessation of plasmapheresis resulted in a rebound of hepatic and renal dysfunction.

In conclusion, cast nephropathy is a rare (or possibly underdiagnosed) entity which results from multiple concurrent insults to the kidney including direct toxicity from bile acids, obstructive physiology from bile casts, and systemic hypoperfusion from vasodilation. It likely represents a broad spectrum of clinical manifestations from initially reversible nephropathy to later irreversible, intractable disease requiring liver and kidney transplantation. The goals of therapy include reduction of bilirubin due to its various mechanisms of renal injury. In patients with cholestatic liver disease, such as patients with primary biliary cirrhosis, primary sclerosing cholangitis, or other liver disease with cholestatic predominance, bile cast nephropathy may be under reported and should be considered in the differential diagnosis. Obtaining a renal biopsy should be considered in patients suspected of this entity to aid in the diagnosis. Although not typical or always available, a trans-jugular approach for renal biopsy may be beneficial as a liver and kidney biopsy may be obtained during the same procedure. When trans-jugular route is not possible, the traditional percutaneous method of obtaining a renal biopsy is sufficient. Patients with hepatorenal dysfunction and the aforementioned characteristic findings on renal biopsy should be evaluated for liver and kidney transplant if more conservative measures are unsuccessful.

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COMMENTS

Case characteristics

A 54-year-old male with past medical history of chronic kidney disease stage 3 and recently diagnosed chronic osteomyelitis of the left heel receiving treatment with Piperacillin-tazobactam presented with complaints of intractable pruritus, anorexia and jaundice.

Clinical diagnosis

He was found to have jaundice, scleral icterus, and a distended abdomen.

Differential diagnosis

His differential diagnosis included acute liver failure, obstructive jaundice, and malignancy.

Laboratory diagnosis

Laboratory evaluation was significant for hyperbilirubinemia, a bland urinalysis with the exception of trace protein and presence of bile, and acute on chronic kidney injury.

Imaging diagnosis

Abdominal ultrasound revealed the presence of mild splenomegaly and ascites, while a CT and MRCP were negative for evidence of biliary obstruction or pathology.

Pathological diagnosis

He was diagnosed with drug-induced liver injury secondary to Piperacillin-tazobactam therapy as well as acute on chronic kidney injury with liver and kidney biopsy indicating stage 1-2 liver fibrosis and bile cast nephropathy, respectively.

Treatment

Initial treatment consisted of medical management consisting of steroids, ursodeoxycholic acid and N-acetylcysteine, followed by dialysis, plasmapheresis, and ultimately liver and kidney transplantation.

Related reports

Bile cast nephropathy is a condition of renal insufficiency as a consequence of hepatic dysfunction that may go unrecognized as it is rarely reported and requires kidney biopsy for definitive diagnosis.

Term explanation

Bile cast nephropathy is a rare and poorly understood condition that occurs in the setting of concomitant renal and hepatic dysfunction largely as a result of hepatic dysfunction.

Experiences and lessons

This entity is likely often under-recognized as it is rare and uncommonly described in the literature. A high index of suspicion for bile cast nephropathy in the setting of renal and hepatic dysfunction should be maintained, and prompt treatment with bilirubin reducing therapy should be initiated as reversal of liver dysfunction improves renal dysfunction.

Peer-review

This is a well-described case report on a rare condition involving concomitant kidney and liver dysfunction including a comprehensive and accurate review of the current literature on a topic which has not previously been well studied. It provides an in-depth explanation of the pathophysiology of bilirubin damage and information pertaining to acute renal injury from toxicity from bile acids with the need for dialysis, plasmapheresis, and ultimately dual organ transplant.

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