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MINIREVIEWS

Expanding the donor pool: Hepatitis C, hepatitis B and human immunodeficiency virus-positive donors in liver transplantation

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Abstract

Liver transplantation (LT) remains the best option for patients with end-stage liver disease but the demand for organs from deceased donors continues to outweigh the available supply. The advent of highly effective anti-viral treatments has reduced the number of patients undergoing LT for hepatitis C (HCV) and hepatitis B (HBV) related liver disease and yet the number of patients waiting for LT continues to increase, driven by an increase in the patients listed with a diagnosis of cirrhosis due to non-alcoholic steatohepatitis and alcohol-related liver disease. In addition, human immunodeficiency virus (HIV) infection, which was previously a contra-indication for LT, is no longer a fatal disease due to the effectiveness of HIV therapy and patients with HIV and liver disease are now developing indications for LT. The rising demand for LT is projected to increase further in the future, thus driving the need to investigate potential means of expanding the pool of potential donors. One mechanism for doing so is utilizing organs from donors that previously would have been discarded or used only in exceptional circumstances such as HCV-positive, HBV-positive, and HIV-positive donors. The advent of highly effective anti-viral therapy has meant that these organs can now be used with excellent outcomes in HCV, HBV or HIV infected recipients and in some cases uninfected recipients.

Key words: Hepatitis C; Hepatitis B; Human immunodeficiency virus; Liver transplantation

Core tip: The optimal utilization of organs from hepatitis C (HCV), hepatitis B (HBV) and human immunodeficiency virus (HIV)-positive donors may help attenuate the current organ shortage. Transplantation of organs from patients with HCV viremia to uninfected recipients can be accomplished safely when coupled with the timely initiation of post-transplant direct-acting antiviral therapy. Suppression of HBV with antiviral

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therapy allows for the safe transplantation from HBV core antibody-positive donors to unexposed recipients, while transplantation of organs from patients who are HBV surface antigen-positive remains investigational. The early experience with HIV-to-HIV positive transplantation via the HOPE act is promising, and allows patients living with HIV improved access to transplantation.

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INTRODUCTION

Approximately 14400 patients are currently awaiting liver transplantation (LT) throughout the United States[1]. Despite an increase in the number of adult liver transplants performed over the past several years, the demand for deceased-donor LT continues to outweigh the available supply of donor organs. While the number of deceased donors has increased slightly, the number of new patients listed for LT continues to increase[2,3]. Furthermore, waitlist mortality remains a concern; of patients who were waitlisted for LT in 2013, only 55% underwent LT 3 years later, while 13% (1362 patients) died and 19% (1991 patients) were removed from the LT list, most commonly for being too ill to undergo transplantation[2]. The increase in waitlist registration appears to be driven by an increase in the number of new listings for patients aged > 65 years, as well as an increase in the proportion of patients listed with a diagnosis of cirrhosis due to non-alcoholic steatohepatitis and alcohol-related liver disease[4,5]. The demand for LT among these patient groups is projected to increase in the future, thus driving the need to investigate potential means of expanding the pool of potential donors. One mechanism for doing so is utilizing organs from donors that previously would have been discarded or used only in exceptional circumstances such as hepatitis C (HCV)-positive, hepatitis B (HBV)-positive, and human immunodeficiency virus (HIV)-positive donors. The advent of highly effective anti-viral therapy has meant that these organs can be used with excellent outcomes in HCV, HBV or HIV infected recipients and in some cases uninfected recipients.

HCV-POSITIVE DONORS

HCV-positive donors encompass donors at any stage of HCV infection (Table 1). This includes patients who are seropositive for anti-HCV antibody (Ab) only (*i.e.*, resolved infection), or those who are HCV-viremic, either in the acute (anti-HCV Ab-negative) or chronic (anti-HCV Ab-positive) phase of infection[6]. The distinction between a viremic donor and one who is seropositive-only is critical when discussing transplantation of an organ from an HCV-positive donor to an uninfected recipient, as the risks of disease transmission differ greatly. While the risk of HCV infection in the recipient approaches 100% when receiving an organ from an HCV-viremic donor, if the donor is only HCV-seropositive and aviremic, the risk of transmission is much lower, ranging from 0-16%[7]. This residual risk of transmission-despite aviremia-is postulated to be due to one of several mechanisms, including interval re-infection among persons who inject drugs (PWID), the presence of low-level viremia, or occult HCV infection in transplanted hepatocytes[7].

In the United States population, HCV-positive donors derive primarily from either the baby boomer birth cohort (born between 1946-1964) or PWID. While baby boomers remain the age group in which HCV prevalence is greatest (2.23% *vs* 1.19% in the general United States population), important demographic shifts are occurring in the epidemiology of HCV[8,9]. A large part of this change is owed to the opioid epidemic, where a high prevalence of injection drug use-especially in Appalachia and the Western United States-has contributed to a tripling of the incidence of HCV infection[9]. In Kentucky, one study suggested a 54.6% prevalence of HCV-seropositivity among a network of PWID. The risk of disease transmission among PWID in these states may be exacerbated by a lack of harm reduction services,

Table 1 Terminology for hepatitis C virus-positive donors

Donor testing	Anti-HCV antibody	HCV RNA
Donor terminology if positive	"Seropositive"	"NAT positive" or "Viremic"
Acute infection	(-)	+
Chronic infection	+	+
Resolved	+	(-)

HCV: Hepatitis C virus; NAT: Nucleic acid test.

including safe injection sites, needle exchanges, and pharmacologic treatment[10]. While HCV incidence and prevalence are increasing among PWID, the number of baby boomers with HCV are in decline due to birth cohort screening and treatment of HCV, but also due to liver related and overall mortality[11,12].

In addition to a high prevalence of HCV infection among PWID, deaths in this population due to opioid overdose have increased. In 2017, there were over 70000 deaths in the United States related to drug overdose, a 9.6% increase from the prior year. The greatest increase in deaths occurred related to synthetic opioids like fentanyl, and occurred in young patients, including those aged between 25-54 years[13]. Given their young age and that many develop hypoxic brain injury before ultimately having brain death declared, many of these individuals may ultimately be evaluated as potential organ donors. Among donors evaluated in 2017, 18% were classified as Public Health Service increase risk donors (IRD), 13.4% had drug intoxication listed as a cause of death, with 8% of these individuals having a history of injection drug use. Among all donors in 2017, HCV-seropositivity was 7.3%, while HCV RNA-positivity was 4.9%; among those who were classified as IRD, HCV-seropositivity and RNA-positivity were 22% and 16%. Taking together both the increased prevalence of HCV in young rural PWID, as well as the young age at which many of these individuals die of overdose-related deaths, the median age of HCV-positive donors has decreased from 48 years in 2010 to 35 years in 2016[6]. One study assessing the utilization of HCV-positive livers in HCV-positive recipients showed that in the era of direct-acting antivirals (DAAs), HCV-positive donors were more likely to be between the ages of 0-30 years, Caucasian, and without a history of diabetes, compared to HCV-positive donors in the pre-DAA era[14].

HISTORICAL USE OF HCV-POSITIVE DONORS

Before the advent of DAAs, transplantation of organs from HCV-positive donors into uninfected recipients could not be considered due to the low efficacy and high risks associated with interferon (IFN)-based therapy in the post-transplant setting. Thus, organs from such patients were reserved for patients with active HCV infection. Because reinfection of the graft is nearly universal regardless of the donor's HCV status, it would seem reasonable to utilize HCV-positive organs for such patients, as they will remain viremic whether they receive an HCV-positive or -negative graft[15]. It should be noted that before 2014, nucleic acid testing (NAT) was not routinely performed on potential donors, so it was generally not possible to know whether the donor was actively viremic and to assess the risk of disease transmission[6]. In older studies, therefore, HCV-positive donors refer only to HCV-seropositive donors.

Early data suggested that this strategy was not associated with impaired outcomes. Of 202 patients with end-stage liver disease (ESLD) related to HCV cirrhosis who underwent LT at a single center from 1992 to 1995, 23 patients received grafts from HCV-positive donors. There was no significant difference in either 1-year or 5-year graft or patient survival, thus supporting the use of organs from HCV-seropositive donors in HCV-infected recipients[16]. A larger study using the United Network for Organ Sharing Scientific Registry of Transplant Recipients confirmed these findings. In this study the outcomes of 96 HCV-infected recipients of HCV-positive organs were compared to those of 2827 patients who received organs from HCV-negative donors. Patient and graft survival were similar and in fact slightly better in the group that received organs from HCV-positive donors (90% *vs* 77% 2-year survival, P = 0.01). This is likely because patients who accepted HCV-positive were less sick at the time of transplantation[17].

Somewhat conflicting data arose from a study published by a group from Europe. In this more recent (but still pre-DAA, IFN-only era) multicenter study, among 694

patients who underwent transplantation for liver disease due to chronic HCV, 11% received organs from HCV-positive donors. When comparing the 63 patients who received HCV-positive organs to 63 controls who received HCV-negative organs, there were no significant differences in patient or graft survival. Secondary outcomes were less favorable, however, with more rapid clinical recurrence of HCV in the HCV-positive donor group, as well as a greater incidence of biliary complications and rejection. Time to recurrence did seem to be shorter in patients who received organs from viremic donors, who comprised 43% of the population of HCV-seropositive donors[18]. Time to post-LT HCV recurrence was also shorter in patients who received grafts that had F1 *vs* F0 fibrosis. The authors concluded therefore, that caution should be exercised in graft selection but that overall there was no detriment to patient or graft survival when transplanting patients with HCV-positive grafts. Given these data, it has been standard of care to offer HCV-positive grafts to HCV-positive recipients for the last 15-20 years.

THE IMPACT OF DAA THERAPY

Despite the promising data showing the essentially neutral effects of utilizing HCV-positive donors for HCV-positive recipients, until the IFN-free DAA era, HCV-positive liver grafts were underutilized and discarded at a high rate. Indeed, 28% of such livers were discarded between 2005 and 2010[14]. In the DAA era, the discard rate has declined to around 11%, owing in large part to a change in physician attitudes regarding the treatment of HCV in the post-transplant setting; as DAAs made treatment easier, there has been an increased acceptance of utilization of HCV-positive livers[14]. Mirroring this, the proportion of HCV-positive recipients who were transplanted with HCV-positive grafts increased, from 6.2% in the IFN era to 16.9% in the DAA era. Such donor-recipient pairings were more common in patients who were on dialysis prior to transplant, those who had a low MELD at listing, and those in a region with relatively lower organ availability[14]. At the center-level, most centers (69%) experienced an increase in utilization of HCV-positive livers.

This increase in utilization and decrease in discard of HCV-positive livers has been driven by the development of DAAs, which have been proven to be safe and effective in the post-LT setting. A number of considerations affecting the use of DAAs, including drug-drug interactions (DDIs) (Table 2) and use in patients with renal dysfunction must, however, be taken into account. Protease inhibitor-based regimens interact in various degrees with calcineurin inhibitors (CNIs), especially cyclosporine. For example, elbasvir/grazoprevir or simeprevir should not be co-administered with cyclosporine due to potentially toxic increases in blood concentrations (increases of 5- to 15-fold) of the protease inhibitors[19]. Co-administration of paritaprevir/ritonavir/ombitasvir + dasabuvir with tacrolimus may lead to a 57-fold increase in the concentration of tacrolimus, which has been shown to lead to significant toxicity in the absence of dramatic dose adjustments[20]. Sofosbuvir-based regimens, including ledipasvir/sofosbuvir (LDV/SOF) and SOF/velpatasvir do not appear to interact significantly with CNI therapy, though there may be some interaction with everolimus leading to increased everolimus trough levels[21]. The primary concern with SOF-based therapy is that SOF is not currently recommended for use in patients with renal dysfunction due to an accumulation of a SOF metabolite of unclear significance. Data from the HCV-TARGET cohort suggest that SOF can be used with high efficacy among patients with renal failure (including those on hemodialysis) but with an increase in anemia, worsening renal function, and other serious adverse events. This suggests that SOF-based regimens may be used in patients with renal dysfunction, albeit with caution[22].

Clinical trial data exist for a number or regimens in the post-transplant setting, including LDV/SOF, daclatasvir and sofosbuvir (DAC+SOF), simeprevir and sofosbuvir (SMV+SOF), and glecaprevir/pibrentasvir (GLE/PIB)[23]. These studies included a majority of genotype (GT) 1 patients, most of whom were treatment experienced, though with varying degrees of fibrosis. Rates of sustained viral response (SVR) were universally high in these studies, except among patients with decompensated cirrhosis post-LT[24-28]. Most recently, a high rate of SVR (97%) was achieved among LT recipients treated with 12 wk of GLE/PIB, a pangenotypic regimen. Importantly, immunosuppression levels did not fluctuate significantly during treatment with GLE/PIB[28]. Further, real-world data from the HCV-TARGET cohort as well as other smaller studies confirm the high rates of SVR and low rates of HCV relapse and adverse events among patients with chronic HCV infection. Predictors of SVR included the absence of cirrhosis and hepatic decompensation, suggesting that treatment earlier in the post-transplant course may be of benefit,

Table 2 Drug-drug interactions among direct-acting antivirals and calcineurin inhibitors

	Cyclosporine (CSA)	Tacrolimus (TAC)	Sirolimus (SRL)	Everolimus (EVR)
Sofosbuvir (SOF)	4.5-fold ↑ in SOF AUC No dose adjustment necessary	13% ↑ in SOF AUC No dose adjustment necessary	Not studied, no interaction expected No dose adjustment necessary	Not studied, no interaction expected No dose adjustment necessary
Ledipasvir	Not studied, no interaction expected	Not studied, no interaction expected	Not studied, no interaction expected	Not studied, may increase EVR concentrations due to mild inhibition of P-gp by ledipasvir
Paritaprevir / ritonavir / ombitasvir + dasabuvir (PrOD)	5.8-fold ↑ in CSA AUC Modeling suggests using 1/5 of CSA dose during PrOD treatment Frequent monitoring necessary	57-fold ↑ in TAC AUC Modeling suggests TAC 0.5 mg every 7 days during PrOD treatment	38-fold ↑ in SRL AUC Do NOT co-administer	27.1-fold ↑ in EVR AUC Do NOT co-administer
Elbasvir / grazoprevir (EBR/GZR)	15-fold ↑ in GZR AUC and 2-fold ↑ in EBR AUC Do NOT co-administer	43% ↑ in TAC AUC No a priori dose adjustment necessary	Not studied, may increase SRL concentrations due to mild inhibition of P-gp by elbasvir	Not studied, may increase EVR concentrations due to mild inhibition of P-gp by elbasvir
Velpatasvir	No interaction observed; no a priori dose adjustment necessary	No data; no a priori dose adjustment necessary	No data; no a priori dose adjustment necessary	Not studied, may increase EVR concentrations due to mild inhibition of P-gp by velpatasvir
Glecaprevir / pibrentasvir (GLE/PIB)	5-fold ↑ in GLE AUC with higher doses (400 mg) of CSA Not recommended in patients requiring stable CSA doses > 100 mg/day	1.45-fold ↑ in TAC AUC No a priori dose adjustment, monitor TAC levels and titrate TAC dose as needed	Not studied, may increase SRL concentrations due to mild inhibition of P-gp by pibrentasvir	Not studied, may increase EVR concentrations due to mild inhibition of P-gp by pibrentasvir
Sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX)	9.4-fold ↑ in VOX AUC Do NOT co-administer	No data; no a priori dose adjustment	Not studied, may increase SRL concentrations due to mild inhibition of P-gp by velpatasvir and voxilaprevir	Not studied, may increase EVR concentrations due to mild inhibition of P-gp by velpatasvir and voxilaprevir

Adapted from www.hcvguidelines.org and www.hep-druginteractions.org. CsA: Cyclosporine; TAC: Tacrolimus; SRL: Sirolimus; EVR: Everolimus; SOF: Sofosbuvir; AUC: Area under the curve; PrOD: Paritaprevir/ritonavir, ombitasvir, and dasabuvir; ELB/GRZ: Elbasvir/grazoprevir; P-gp: P-glycoprotein; VOX: Voxilaprevir; VEL: Velpatasvir.

before these complications develop[29]. Based on the available data, guidance in the United States recommend treatment with 12 wk of various regimens depending on HCV genotype and the presence of (decompensated) cirrhosis[30].

RATIONALE AND EARLY DATA FOR TRANSPLANTATION OF LIVERS FROM HCV-POSITIVE DONORS TO HCV-NEGATIVE RECIPIENTS

DAA therapy has allowed for safe and highly effective treatment of HCV infection in LT recipients. Because of the high efficacy of these treatments in the pre-LT setting as well, the number of patients placed on the LT waiting list for liver disease related to HCV infection has been in decline since 2016[31] while the number of HCV-positive donors is on the rise. These donors are mostly young people dying of causes unrelated to their HCV infection, and therefore may be good candidates for organ donation. If organ quality is good, and the risks related to post-LT HCV infection can be eliminated by prompt and effective antiviral therapy, then it would be ethically questionable to withhold the transplantation of such organs to sick patients awaiting LT.

Early case reports suggested the overall safety of this approach. Three patients received organs (2 kidney recipients and 1 liver recipient) from a high-risk donor who was HCV NAT test negative, but recently had sexual contact with an HCV-infected male partner. The donor was likely in the eclipse phase of HCV infection, prior to detectable viremia, and transmitted HCV infection to all three recipients. All recipients were treated with DAA therapy, and all achieved SVR without adverse effects on their graft[32]. Another case report described the utility of using an HCV-viremic organ in an uninfected recipient who had multiple complications of portal hypertension but low priority on the LT waiting list and had no potential living donors. The recipient rapidly became viremic at 3-d post-LT and was ultimately treated starting on post-operative day 25 with a 24-wk course of LDV/SOF, and

successfully achieved SVR with no adverse effect on the graft[33].

Further proof of this concept was demonstrated in the context of renal transplantation in the THINKER trial where 10 patients who had long anticipated waiting times accepted kidneys from HCV-viremic donors. All donors were known to be GT 1 prior to transplantation, and all recipients received elbasvir/grazoprevir for a 12-wk course when viremia was detected in the recipient. All recipients developed HCV viremia on day 3 post-transplantation and were started on treatment immediately; all achieved SVR without significant changes in kidney or liver function[34]. Further follow-up demonstrated good 1-year outcomes in the initial patient population, as well as 6-month outcomes for an additional 10 patients with good long-term renal and quality-of-life outcomes[35]. More recently, in an open-label trial in heart transplant recipients, pangenotypic antiviral therapy with GLE/PIB was provided pre-emptively to 20 recipients of hearts from NAT-positive donors. All patients tolerated treatment well and achieved SVR[36].

In the context of LT, modeling data suggests that for any HCV-uninfected patient with decompensated cirrhosis awaiting LT, accepting any liver (HCV-positive or -negative) is associated with a survival benefit compared to accepting only HCV-negative organs once the recipient's MELD score exceeds 20. This was noted to be the case irrespective of geographic location or prevalence of HCV-positivity among the donor population[37] and was cost effective compared to restricting acceptance to HCV-negative livers only at a recipient MELD score of 22[38]. This is an important finding as one potential complication of transplanting HCV-viremic organs into uninfected recipients could be a lack of insurer coverage for DAA treatment, leaving the patient with the potential for complications of a newly acquired HCV infection in an immunocompromised state.

More recent data suggests a growing acceptance of this practice. Kwong *et al*[39] reported the transplantation of 10 HCV-uninfected recipients with liver grafts from HCV-viremic donors. These grafts were offered to patients with a high estimated risk of waitlist dropout, including those with hepatocellular carcinoma. All recipients developed HCV viremia on day 4 post-LT. Contrary to the THINKER trial, which was an industry-sponsored study, in this study providers were required to obtain insurance approval for each patient prior to initiation of therapy, just as if the patient were being treated in any other clinical context. Therefore, treatment was not initiated until a median time of 43 d. Treatment regimen was at the discretion of the provider, and consisted of SOF-based therapies and all patients achieved SVR[39]. Adverse events included 1 patient who developed leukopenia and anemia and 3 patients who developed biopsy-proven rejection. Recurrent HCV was not seen in any of the allografts. Two of the patients developed rejection within 1 month of LT, prior to initiation of HCV treatment (one with both acute cellular rejection and antibody-mediated rejection, the other with only acute cellular rejection), and one developed antibody-mediated rejection 5 mo after transplant, after completing HCV treatment. Immunosuppression levels did not vary appreciably to explain the development of rejection in these patients, though it is possible that either HCV infection itself or treatment with DAAs may have led to some immunologic changes that increased the risk of rejection in this population. The authors concluded that it is difficult to draw conclusions given the small sample size, and that this connection should be further investigated among HCV-uninfected patients who receive HCV-viremic grafts[39].

Another recent study by Cotter and colleagues examined the practice of transplantation from HCV-seropositive and/or -viremic donors to HCV-uninfected recipients from January 2008 to January 2018 in the United States (Table 3). During this time, there were 2635 transplants performed with using HCV-seropositive livers, of which 2378 were given to 2378 HCV-seropositive recipients. The number of HCV-seropositive to -negative transplants increased from 7 in 2008 to 107 in 2017, or from 55 in the pre-DAA era to 202 in the post-DAA era. HCV-uninfected patients who received -seropositive livers had higher MELD scores and waitlist times, and received livers from younger and lower body-mass index donors[40]. Three-year graft survival in the DAA era was essentially equivalent at 85.1% compared with 84.5% among patients who received HCV-seropositive versus -negative grafts. Similar results were seen in HCV-viremic donor to HCV-uninfected recipient transplants with no difference in 2-year graft survival among recipients of grafts from HCV-viremic donors compared to HCV-aviremic donors[40].

RISKS ASSOCIATED WITH POST-LIVER TRANSPLANT HCV INFECTION

There is still a concern that acute HCV in the post-transplant setting can be severe,

Table 3 Graft survival is similar in HCV-negative recipients of livers from HCV NAT-positive or -negative donors (Data from Cotter *et al*[40])

	1-yr	2-yr
DNAT-/R-	93%	88%
DNAT-/R+	93%	88%
DNAT+/R-	93%	86%
DNAT+/R+	94%	90%

DNAT: Donor HCV NAT status; R: Recipient HCV NAT status.

especially if there is a delay in initiating treatment with DAAs. Effective and timely treatment for HCV-infected individuals post-LT is essential as the course of HCV is accelerated in the post-transplant setting, with up to 30% of patients developing cirrhosis within 5 years of LT. In addition, up to 9% of patients may develop a severe form of HCV, fibrosing cholestatic hepatitis (FCH), with a very high viral load, progressive cholestasis and early graft loss. With DAAs, progression of FCH can be aborted, with data from a number of studies suggesting rates of SVR ranging from 73%-100%[24,25,41,42]. In the IFN era, these complications made it such that LT for HCV-related cirrhosis was associated with the worst outcomes post-LT compared with other etiologies of liver disease[21]. In the current era, however, post-LT survival has improved significantly for patients who undergo LT for HCV, equivalent to that of recipients transplanted for etiologies other than HCV[43].

One potential consequence of effective HCV treatment is the development of immune-mediated graft dysfunction (IGD). IGD was seen in approximately 7.2% of LT recipients treated with IFN-based therapies and was characterized predominantly by the development of plasma cell hepatitis and was associated with lower long-term survival (61.5% *vs* 91.3%) compared to patients without IGD[44]. IGD appears to be less common following DAA therapies, occurring with a rate of 3.4%. While the mechanism for IFN-associated IGD is likely related to an augmentation of the immune response, the mechanism driving IGD in patients treated with DAAs is less clear[45]. Patients should be monitored closely for the development of rejection during treatment with DAAs, especially among HCV-uninfected recipients receiving grafts from viremic patients.

Extrahepatic complications that must be monitored for in the post-LT setting in untreated patients include new-onset diabetes mellitus, glomerulonephritis, and lymphoproliferative disorders. While most patients are at risk for the development of DM in the post-transplant setting owing to the metabolic effects of calcineurin inhibitors, the presence of concomitant chronic HCV infection increases that risk, with a prevalence ranging from 13 to 28%[46]. Along with its metabolic effects, HCV contributes to post-LT renal dysfunction through a variety of mechanisms, in some cases via induction of cryoglobulinemia or HCV-associated glomerulonephritis. Finally, HCV is an independent risk factor for the development of lympho-proliferative disorders, including non-Hodgkin lymphomas[46]. With timely antiviral therapy, the occurrence of these complications may be limited; however, it is critical to consent patients who may be interested in receiving HCV-seropositive or -viremic donor livers for these risks in the even that antiviral therapy is delayed.

HBV

Prior to effective anti-viral therapy, recurrence of HBV after LT for HBV related liver disease was a feared complication with high rates of allograft failure and mortality[47,48]. The use of hepatitis B immune globulin (HBIG) as passive immunization after LT dramatically reduced the risk of recurrent HBV and improved survival[49], and the addition of anti-virals such as lamivudine further reduced the risk of HBV recurrence such that long term survival after LT is better than most other indications[50]. The current strategy to prevent HBV recurrence after LT consists of indefinite oral anti-viral therapy with or without HBIG, with most centers in the United States using only a very short course (less than 3 mo) of HBIG.

Unlike the situation with HCV-infection where DAA therapy is a cure, current therapy for chronic HBV-infection [defined as patients with persistently positive HBV surface antigen (HBsAg)] aims to suppress viral replication. Chronic HBV infected patients can be further defined by the presence or absence of HBV envelope antigen (HBeAg) as either HBeAg positive or negative. In the non-immunosuppressed patient therapy can be finite if HBeAg positive patients develop durable HBeAg negativity and the development of positive anti-HBe with a negative HBV DNA. However, in

HBeAg negative patients therapy is indefinite as it needs to be in the immunosuppressed patient as there is a very high risk of flare of HBV when therapy is withdrawn.

HBV core antibody positive donors

The virology of HBV is complex and complete clearance of virus after infection is difficult to achieve with current therapies. The reactivation of HBV after chemotherapy is well recognized and in the United States guidelines from the American Society of Clinical Oncology recommend starting antiviral therapy for HBsAg-positive/anti-HBc-positive patients before or with chemotherapy and monitoring HBsAg-negative/anti-HBc-positive patients for reactivation with HBV DNA and ALT levels, starting antivirals if reactivation occurs but in those undergoing chemotherapy associated with a high risk of HBV reactivation antivirals can be started pre-emptively[51]. Much of the concern over chemotherapeutic regimens and reactivation of HBV has occurred recently with the advent of biologic therapies with direct effects on immunity. The original reports of HBV reactivation from immunosuppression came from the transplant arena more than 20 years ago.

The National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database examined 674 LT recipients and their donors for evidence of transmission of HBV between 1989 and 1994[52]. Of the 23 HBV-negative recipients of livers from anti-HBc positive donors, 18 (78%) developed HBV infection with appearance of HBsAg even though donors had been HBsAg negative, with reduced survival. This time period coincided with the use of HBIG and in small series it appeared to be effective in preventing HBV infection in recipients of anti-HBc positive live donor allografts[53].

The introduction of lamivudine further improved the survival of recipients of anti-HBc positive donors. In a United States study of 15 patients (6 who were HBsAg positive and 9 who were HBsAg negative at time of LT) who received anti-HBc positive allografts were followed for a mean of 17 mo. All patients received lamivudine daily and HBIG was given to HBsAg positive patients. All 15 patients remained HBsAg negative and 9 underwent liver biopsy after LT with only 1 patient having detectable HBV DNA in liver tissue (although remained HBsAg negative and anti-HBs positive)[54]. Similar results were noted in a Taiwanese cohort of 16 recipients of anti-HBc positive live donor liver allografts with no evidence of de novo HBV infection after a mean follow up of 25 mo[55].

Despite the success of antiviral therapy there has been some controversy when examining long-term outcomes. A large prospective observational Italian study of 219 LT recipients who received anti-HBc positive deceased donor allografts between 2007-2009 suggested that recipients who were HBsAg positive who received these organs had an increased 3 year survival compared to recipients who were HBsAg negative[56]. Interestingly only 1 patient developed graft loss due to de novo HBV infection suggesting that other factors were responsible for the decreased survival in HBsAg negative recipients. However, good long-term survival was demonstrated in 64 HBsAg negative recipients of anti-HBc positive allografts with 69% 5-year survival using a regimen of HBIG at the time of LT and then daily lamivudine[57]. Nine patients developed de novo HBV infection despite this prophylaxis but were successfully treated with adefovir or tenofovir. Even better results have been seen in the pediatric population with 92% 10-year survival in 41 recipients of anti-HBc positive allografts using a combination of HBIG for 1 year post-LT and yearly HBV vaccine, without antivirals[58].

HBV surface antigen positive donors

With the continued organ shortage every effort should be made to use donor liver allografts that previously may have been discarded. This is particularly the case in areas of the world where HBV infection is endemic and the prevalence of anti-HBc positivity can be as high as 80%. The encouraging results using these types of liver donors with highly effective anti-viral therapy has led to the possibility of using donors who are HBsAg positive and therefore likely to have chronic HBV infection.

Several reports have emerged demonstrating that HBsAg positive deceased donors can be safely used in HBsAg positive or HBsAg negative recipients. A small Italian study of 10 patients followed for a median of 42 mo after LT using HBsAg positive donors with HBIG and antiviral therapy and showed no evidence of HBV hepatitis in any patient with half of HBsAg negative recipients remaining HBsAg negative after LT[59]. A larger study in Asia compared 42 adult recipients of HBsAg positive donors with 327 patients who received HBsAg negative donors and noted comparable graft and patient survival[60]. All the recipients of HBsAg positive allografts remained HBsAg positive without evidence of HBV hepatitis and were mainly receiving oral antiviral therapy without HBIG. Closer examination of viral activity suggests that

there is low level viremia early on after LT with HBsAg positive donors but this becomes undetectable within a few months[61].

In the United States the American Society of Transplantation published consensus guidelines regarding the use of HBV positive donors but only refers to anti-HBc positive allografts and suggests that these donors should be considered for all adult transplant candidates with lamivudine as the antiviral prophylaxis of choice without HBIG[62]. Hence the use of HBsAg positive donors needs further investigation.

HUMAN IMMUNODEFICIENCY VIRUS

The advent of highly effective anti-retroviral therapy (HAART) for HIV infection in the mid 1990s meant that a previously fatal disease was now a chronic illness. Patients with HIV infection share some of the risk factors for acquiring viral hepatitis infection and it became clear that rather than dying of AIDS, liver disease was becoming the leading cause of death in HIV patients, mainly from HBV or HCV infection[63,64].

Early reports

The first reports of LT in HIV patients were in carefully selected patients with only short term follow up. Norris *et al* reported on 14 HIV-infected liver allograft recipients (7 with HCV infection, 7 non-HCV) transplanted over 8 years in a single institution[65]. All the patients in the non-HCV infected cohort were alive at 1-year follow up but 4 of the HCV group died of complications from recurrent HCV infection and sepsis, despite HAART in the majority. Further reports confirmed that short-term outcomes were acceptable in patients with stable HIV after LT (91% at 1 year) but recurrent HCV infection was very common and affected patient and graft survival, decreasing to 64% at 3 years[66]. The National Institutes of Health (NIH) Solid Organ Transplantation in HIV trial enrolled 232 patients with HIV infection who underwent primary LT over 12 years and compared them to non-HIV infected patients (with and without HCV infection) transplanted over the same time frame in the United States. Of these 232 patients, 72 had HIV mono-infection and 160 had HIV/HCV co-infection. The presence of HCV infection increased the risk of post-LT mortality with a hazard ratio of 1.46 in HCV mono-infected and 2.62 in HCV/HIV co-infected patients whereas HIV mono-infection did not affect post-LT mortality[67]. Hence HIV patients could successfully undergo LT but recurrent HCV infection leading to allograft failure was the main determinant of long-term survival since interferon based therapy was largely ineffective and not well tolerated.

The advent of direct acting anti-viral agents (DAA) has transformed the therapy of HCV infection and cure rates of almost 100% are common. Similar success has been reported after LT in both HCV mono- infected and HCV-HIV co-infected patients without significant side effects meaning recurrent HCV infection after LT can be treated or prevented in HIV patients that should lead to good long-term outcome[68].

HOPE act

Up until 2013 federal law prohibited the use of organs from deceased donors with HIV infection. Worldwide, there is a shortage of deceased donor organs and patients with HIV infection have higher wait-list mortality. Several countries with high HIV infection rates among the general population demonstrated that HIV infected donors could be an important source of deceased donor organs with excellent outcomes[69]. In Europe reports emerged of long-term success of HIV-positive donors to HIV-positive recipients with undetectable HIV viremia on HAART[70]. Eventually the HIV Organ Policy Equity (HOPE) Act was passed by the United States Congress in November 2013 allowing the use of HIV positive donors in HIV positive recipients.

Initial reports have been encouraging with several centers performing transplants under research protocol with excellent results since the first HIV positive donor to HIV positive recipient in March 2016 at Johns Hopkins[71]. Guidelines have also been developed by the American Society of Transplantation regarding solid organ transplantation in HIV-infected recipients but await more data before making any firm recommendations for HIV-positive donors[72]. A recent survey of transplant centers in the United States suggested that most were aware of the research restrictions of the HOPE Act that the use of HIV positive donors should be under protocol and supported this policy. In addition, the local HIV prevalence, HIV positive recipient volume, overall transplant volume and increased infectious rick donor utilization were important determinants of whether centers were planning HIV positive donor to HIV positive recipient transplants[73].

An unexpected benefit of the HOPE Act has been the utilization of organs from deceased donors that would previously have been discarded as they were thought to be from HIV-positive donors although this was the result of a false-positive HIV

screening test. This was examined in the HOPE in Action trial where donors who tested positive for anti-HIV antibody or HIV nucleic acid test but were not known to have HIV infection were classified as false-positive donors. From these 10 suspected false positive donors, 21 HIV-positive recipients were transplanted, including 5 liver and one liver-kidney recipient. All of the donors were subsequently shown to be HIV-non-infected. Extrapolating these results to all donors in the US, 50-100 false positive HIV donors can be expected[74].

Unlike the situation with HCV positive donors, at this time the use of HIV-positive donors to HIV-negative recipients cannot be advocated. The almost universal cure rate of current HCV therapy means that HCV-negative recipients of HCV positive liver allografts are almost guaranteed to clear the HCV infection after transplantation. A recent report described a live donor LT from an HIV-positive mother to her HIV-negative child in South Africa as a life-saving measure. Using pre-operative HIV-prophylaxis in the child, HIV infection in the child has not been observed after more than a year after transplantation[75].

CONCLUSION

The high efficacy and safety of antiviral therapy for the treatment of viral hepatitis has provided the transplant community with the opportunity to utilize organs from donors infected with HCV and HBV and these infections can be easily treated after LT. The HOPE Act in the United States has allowed the transplantation of organs from HIV-positive donors into HIV-positive recipients that previously would have been discarded. In the case of HCV, the almost 100% cure rates of DAA therapy means that HCV-positive organs can be considered for those patients on the LT waiting list not currently infected with HCV. Due at least in part to the tragic effects of the opioid epidemic in the United States, HCV-positive, HBV-positive and HIV-positive donors are increasing in prevalence and come from younger people, a demographic associated with very favorable long-term outcome after LT. The success of DAA therapy even in HCV-infected cirrhotic patients means that HCV-related liver disease is declining as an indication for LT, and many of the sickest patients awaiting LT may be HCV-negative. The use of HBV-positive and HIV-positive organs in HBV-positive and HIV-positive recipients is an efficient method of utilizing organs that otherwise would be discarded. The use of these organs in HBV-negative or HIV-negative recipients is still not advised unless in highly exceptional circumstances as these infections can currently only be suppressed and not cured. Modeling and real-world data so far suggest that the practice of transplanting organs from HCV-positive donors into HCV-negative recipients is associated with good short-term outcomes and is becoming standard practice at many centers. Longer term data is needed to fully assess the effects of this practice.

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ORIGINAL ARTICLE

Basic Study

Non-SMC condensin I complex subunit D2 and non-SMC condensin II complex subunit D3 induces inflammation *via* the IKK/NF-κB pathway in ulcerative colitis

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Abstract

BACKGROUND

Ulcerative colitis (UC) is a chronic, nonspecific intestinal inflammatory disease with undefined pathogenesis. Non-SMC condensin I complex subunit D2 (NCAPD2) and non-SMC condensin II complex subunit D3 (NCAPD3) play pivotal roles in chromosome assembly and segregation during both mitosis and meiosis. To date, there has been no relevant report about the functional role of NCAPD2 and NCAPD3 in UC.

AIM

To determine the level of NCAPD2/3 in intestinal mucosa and explore the mechanisms of NCAPD2/3 in UC.

METHODS

Levels of NCAPD2/3 in intestinal tissue were detected in 30 UC patients and 30 healthy individuals with in situ hybridization (ISH). *In vitro*, NCM60 cells were divided into the NC group, model group, si-NCAPD2 group, si-NCAPD3 group and si-NCAPD2+si-NCAPD3 group. Inflammatory cytokines were measured by ELISA, IKK and NF-κB were evaluated by western blot, and IKK nucleation and NF-κB volume were analyzed by immunofluorescence assay.

RESULTS

guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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Compared with expression in healthy individuals, NCAPD2 and NCAPD3 expression in intestinal tissue was significantly upregulated ($P < 0.001$) in UC patients. Compared with levels in the model group, IL-1β, IL-6 and TNF-α in the si-NCAPD2, si-NCAPD3 and si-NCAPD2+si-NCAPD3 groups were significantly downregulated ($P < 0.01$). IKK and NF-κB protein expression in the si-NCAPD2, si-NCAPD3 and si-NCAPD2+si-NCAPD3 groups was significantly decreased ($P < 0.01$). Moreover, IKK nucleation and NF-κB volume were suppressed upon si-NCAPD2, si-NCAPD3 and si-NCAPD2+ si-NCAPD3 transfection.

CONCLUSION
NCAPD2/3 is highly expressed in the intestinal mucosa of patients with active UC. Overexpression of NCAPD2/3 promotes the release of pro-inflammatory cytokines by modulating the IKK/NF-κB signaling pathway.

Key words: Non-SMC condensin I complex subunit D2; Non-SMC condensin II complex subunit D3; Ulcerative colitis; Inflammation; IKK/NF-κB; Pathway

Core tip: In this study, non-SMC condensin I complex subunit D2 (NCAPD2) and non-SMC condensin II complex subunit D3 (NCAPD3) expression levels have been confirmed to be significantly up-regulated in the intestinal mucosa of patients with active UC. *In vitro*, the data suggested that silencing NACPD2 and NACPD3 could depress the expression of IL-1β, IL-6 and TNF-α. Further, knockdown of NACPD2 and NACPD3 could remarkably suppress IKK nucleation and NF-κB volume. These results suggest that NACPD2 and NACPD3 are over-expressed in the intestinal mucosa of patients with UC, and overexpression of NCAPD2/3 promotes the release of pro-inflammatory cytokines by modulating the IKK/NF-κB signaling pathway.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, nonspecific intestinal inflammatory disease with undefined pathogenesis, and includes ulcerative colitis (UC) and Crohn's disease[1]. The course of IBD is prolonged and repetitive. Although there are many medical treatments for the disease, the effects of treatment are unsatisfactory and the prognosis is poor, which seriously affects patient quality of life, exhausts many health resources and is one of the precancerous lesions of intestinal tumors[2,3].

One of the biggest problems in the diagnosis of IBD is the differential diagnosis of infectious intestinal disease, with the most difficult being the differential diagnosis of intestinal tuberculosis[4]. In recent years, many reports have found that serological markers have great value in the differential diagnosis of IBD. Therefore, the search and development of clinical biomarkers that can accurately diagnose IBD and monitor the status of IBD disease progression have important significance and value for improving the clinical treatment of IBD patients[5,6].

Non-SMC condensin I complex subunit D2 (NCAPD2) and non-SMC condensin II complex subunit D3 (NCAPD3) play key roles in chromosomal structural changes and separation during the process of eukaryotic cell mitosis[7,8]. NCAPD2 and 3 are subunits of condensin I and condensin II, respectively. Condensin is of vital importance in the contraction and separation of chromosomes during eukaryotic cell mitosis. When NCAPD2 and NCAPD3 function abnormally, abnormal chromosome structure can occur, leading to mitotic cell abnormalities. Studies have shown that the NCAPD2 gene is associated with Parkinson's disease in Han Chinese people, and is correlated with gender and age[9]. Patients with high NCAPD3 expression have a lower postoperative recurrence rate after receiving prostatic cancer surgery. NCAPD3

can be used as a postoperative prognostic indicator of prostatic cancer[10]. Yin *et al*[11] found that high expression of NCAPH, a NCAPD3 homologous complex, promotes colonic cancerous cell proliferation. It has been reported that NCAPD3 plays an important role in microbial immunity and in the process of human intestinal epithelial cells to clear bacteria[12]. In this study, the role and possible mechanisms of NCAPD2/3 in the development of UC was explored.

MATERIALS AND METHODS

Clinical samples and in situ hybridization

Paraffin-embedded intestinal tissue specimens from colonoscopies were collected from 30 patients with active UC (Mayo endoscopic score > 2) and 30 age- and sex-matched healthy people (Mayo endoscopic score = 0) between October 2016 and September 2017. These samples were analyzed retrospectively using protocols approved by the ethics committee of the Affiliated Hospital of Nanjing University of Chinese Medicine (2018NL-170-02). The information regarding the tissue samples used for in situ Hybridization (ISH) in this study is provided in Table 1. As a retrospective study using discarded tissue, the ethics committee agreed to the use of these samples and waived informed consent.

Paraffin sections were routinely dewaxed with water, and 50 μL of levamisole was added, incubated at room temperature for 30 min and washed with high pressure water three times for 3 min each. Then, 50 μL of 1 × proteinase K was added, incubated at 37 °C for 20 min and washed with distilled water once for 3 min. For prehybridization, pre-hybrid solution was placed on each slide and incubated at 42 °C for 2-4 h. The excess liquid was drained, and 50 μL probe hybridization solution was added to each slide (NCAPD2 and NCAPD3 were purchased from Wuhan Boster Biological Technology Company) and hybridized at 42 °C overnight. SSC liquid elution was performed in a 37 °C water bath using the following parameters: 2 × SSC wash for 5 min twice, 0.5 × SSC wash for 15 min twice, and 0.2 × SSC wash for 15 min twice. Then, slides were incubated in 50 μL 1 × sealed protein solution at 37 °C for 30 min, and excess liquid was drained off without washing. The slides were incubated with 50 μL of rabbit anti-digoxin (1:100 dilution) at 37 °C for 1 h, followed by incubation with 50 μL of AP-sheep anti-rabbit IgG (1:100 dilution) at 37 °C for 1 h. NBT/BCIP was used for color development, and the reaction was stopped at any time by microscopy. Gradient ethanol was used for dehydration, xylene for transparence, and neutral gum to seal the slides. The probe-containing hybridization solution was replaced by a probe-free hybridization buffer solution for the negative control. Image-ProPlus 6.0 image analysis software was used for image analysis, and the average OD was calculated. Then, the average view value was used as the expression quantity of the specimen.

Cell culture and groupings

After normal recovery, NCM460 cells (ATCC, United States), which are normal colonic epithelial cells, were cultured in a thermostatic incubator using RPMI 1640 culture medium (37 °C, 5% CO_2). NCM460 cells were divided into the normal control group (NC group), model group, si-NCAPD2 transfection group (si-NCAPD2 group), si-NCAPD3 transfection group (si-NCAPD3 group) and si-NCAPD2 plus si-NCAPD3 group (si-NCAPD2+si-NCAPD3 group). The NC group cells were cultured in normal culture medium. After transfection with si-NCAPD2 and si-NCAPD3 (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), the si-NCAPD2, si-NCAPD3 and si-NCAPD2+si-NCAPD3 groups, along with the model group, were stimulated with 50 ng/mL of LPS (Sigma, United States).

ELISA detection

After 48 h of cell culture, cell culture medium from each group was collected. IL-1β, IL-6 and TNF-α levels in the culture medium of each group were detected according to the instructions for the IL-1β, IL-6 and TNF-α kits (Sigma, United States).

Western blot assay

The cells were lysed using RIPA lysis buffer to collect protein. The total protein concentration was determined by BCA colorimetry assay. Then, 40 μg of protein was loaded into each well of the gel. After electrophoresis and transfer of proteins from the gel to a membrane, the membrane was blocked with 5% nonfat dried milk. The membrane was washed with TBST three times, and NCAPD2 (Abnova, Taiwan, China) (1: 1000), NCAPD3 (Proteintech Group, United States) (1:1000), IKK, NF-κB (Abeam, Cambridge, United Kingdom) (1:1000) and GAPDH antibodies (1: 1000) were added and incubated overnight at 4°C. After the membrane was washed with TBST

Table 1 Patient characteristics

	Normal control, *n* = 30 (%)	Ulcerative colitis[1], *n* = 30 (%)
Age in yr		
A2 (18-40)	16 (53.3)	17 (56.7)
A3 (≥ 40)	14 (46.7)	13 (43.3)
Sex		
Male	15 (50.0)	14 (46.7)
Female	15 (50.0)	16 (53.3)
Tissue sampling site		
Transverse colon	2 (6.7)	3 (10.0)
Descending colon	7 (23.3)	6 (20.0)
Sigmoid colon	13 (43.3)	9 (30.0)
Rectum	8 (26.7)	12 (40.0)
Therapy		
5-ASA	N/A	13 (43.4)
Corticosteroids	N/A	4 (13.3)
5-ASA/corticosteroids	N/A	3 (10.0)
Immunomodulators/corticosteroids	N/A	1 (3.3)
Chinese herbal medicine	N/A	5 (16.7)
No treatment	N/A	4 (13.3)

[1]All patients included in this study had active disease, and tissue inflammation was scored as moderate/severe (Mayo endoscopic score > 2). 5-ASA: 5-aminosalicylic acid; N/A: Not applicable.

three times, the second antibody (1:1000) was incubated for 1 hour and ECL was used for image development. Protein gray values in each group were read.

Immunofluorescence staining

After being treated for 48 h, each group of cells was fixed with 4% paraformaldehyde for 30 min, ruptured with 0.01% Triton X-100 for 10 min, and blocked with 0.1% bovine serum albumin for 30 min. After washing with PBST, diluted primary IKK or NF-κB antibody (Abcam, Cambridge, United Kingdom) was added and incubated overnight at 4°C. Sheep anti-rabbit IgG-Cy3 (1:50) and sheep anti-mouse IgG-FITC (1:50) secondary antibodies were added and incubated at room temperature in the dark for 30 min. The nuclei were stained with DAPI, and the slides were sealed with glycerol. The staining results were observed under a laser scanning confocal microscope. Image analysis software IPP 6.0 was used to analyze the surface density, optical density, and number of positive cells in each image.

Statistical analysis

All numerical data were processed with SPSS 22.0. One-way analysis of variance was used for inter-group comparisons. Experimental data are expressed as the mean ± SD. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Clinical and analysis

Mucosal samples were taken during colonoscopy from inflamed rectal or colonic mucosa of 30 patients with active UC, as well as of 30 age- and sex-matched controls (Table 1). The patients with active UC were defined as having a Mayo endoscopic score > 2. Location of the samples acquired and the ongoing treatment are shown in Table 1. Compared with expression in healthy individuals, NCAPD2 and NCAPD3 expression in intestinal tissues of UC patients was significantly increased ($P < 0.001$) by ISH assay. The relevant data are shown in Figure 1.

Inflammatory cytokine expression in different groups in vitro

ELISA results showed that the IL-1β, IL-6 and TNF-α concentrations of the Model and si-Ctrl groups were significantly upregulated compared with those of the NC group ($P < 0.01$). Upon si-NCAPD2 or/and si-NCAPD3 transfection, the IL-1β, IL-6 and TNF-α concentrations of the si-NCAPD2, si-NCAPD3 and si-NCAPD2+ si-NCAPD3

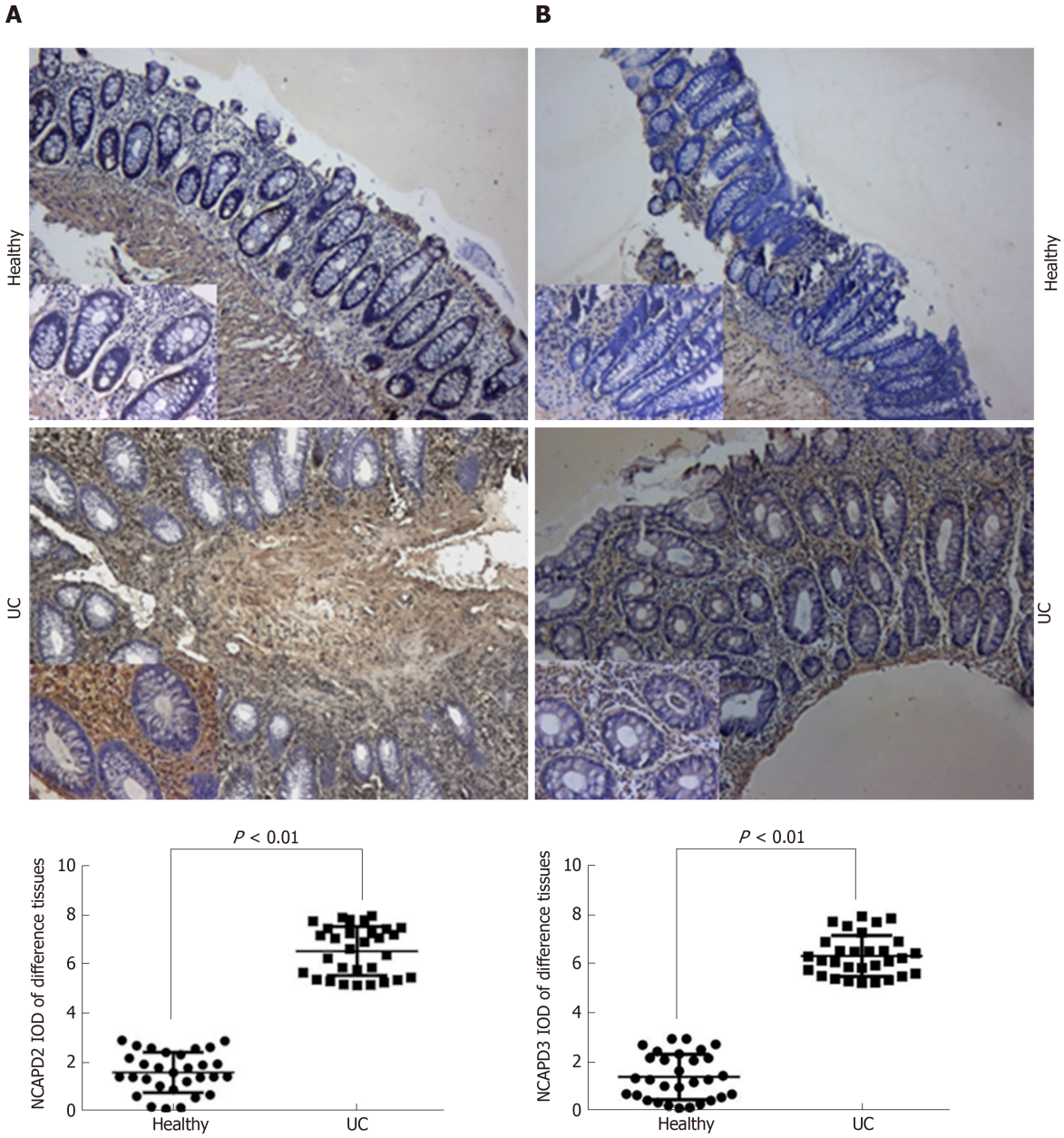

Figure 1 Non-SMC condensin I complex subunit D2 and non-SMC condensin II complex subunit D3 expression in intestinal tissues. A: Non-SMC condensin I complex subunit D2 expression by *in situ* hybridization assay (× 100, × 400); B: Non-SMC condensin II complex subunit D3 expression by *in situ* hybridization assay (× 100, × 400). UC: Ulcerative colitis; NCAPD2: Non-SMC condensin I complex subunit D2; NCAPD3: Non-SMC condensin II complex subunit D3.

groups were significantly decreased compared with those of the Model group ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively). The relevant data are shown in Figure 2.

Relative protein expression levels by western blot assay

To evaluate relative protein expression, we measured the expression of NCAPD2, NCAPD3, IKK and NF-κB by western blot (WB) assay. The results showed that NCAPD2, NCAPD3, IKK and NF-κB protein expression in the Model and si-Ctrl groups was significantly increased compared with that of the NC group ($P < 0.001$). Moreover, NCAPD2 protein expression in the si-NCAPD2 and si-NCAPD2+ si-NCAPD3 groups was significantly suppressed compared with that of the Model group ($P < 0.01$). NCAPD3 protein expression in the si-NCAPD3 and si-NCAPD2+ si-NCAPD3 groups was significantly suppressed compared with that of the Model group ($P < 0.01$). Protein expression of IKK and NF-κB in the si-NCAPD2, si-NCAPD3 and si-NCAPD2+si-NCAPD3 groups was significantly downregulated compared with that of the Model group ($P < 0.01$ and $P < 0.001$, respectively). The relevant data are shown in Figure 3.

IKK and NF-κB protein expression by immunofluorescence

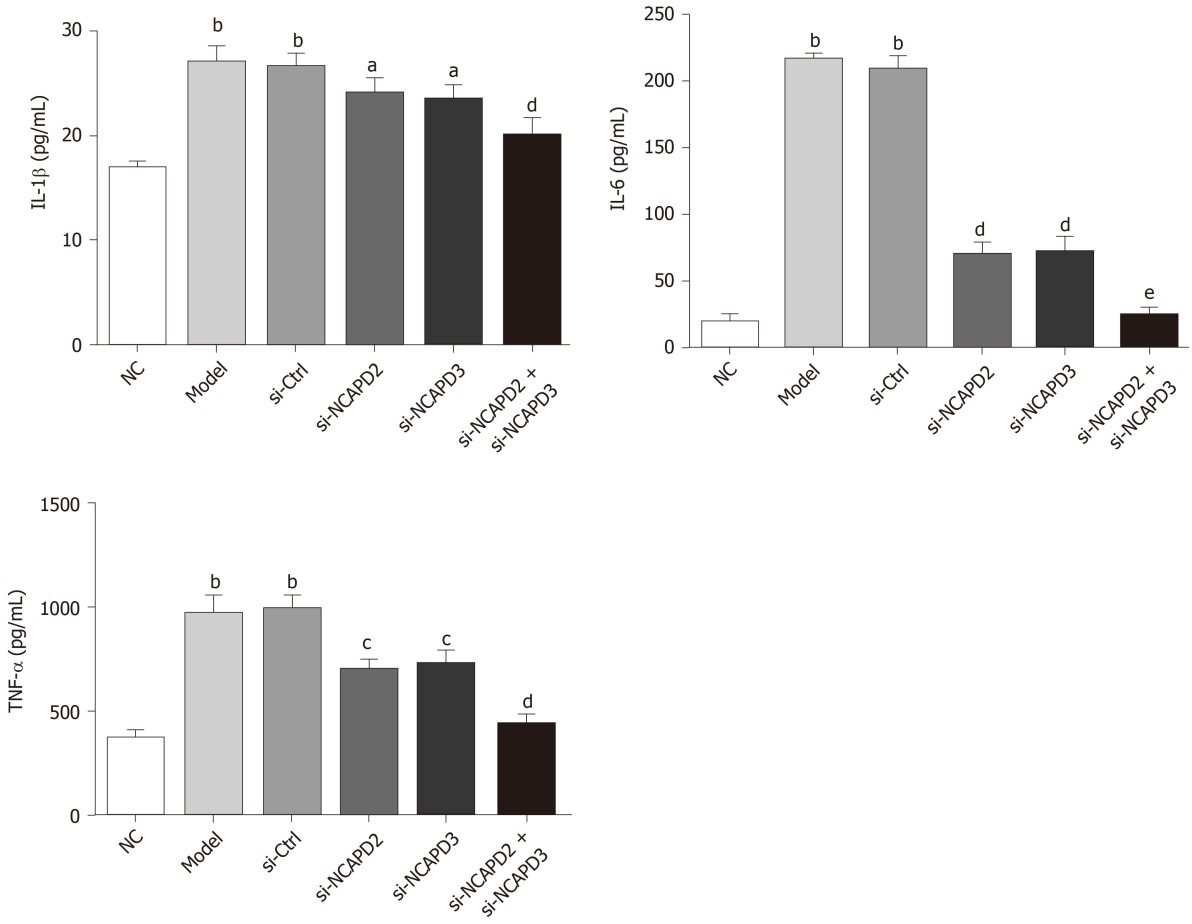

Figure 2 Inflammatory cytokines of different groups. $^{b}P < 0.01$, *vs* NC; $^{c}P < 0.05$, $^{d}P < 0.01$, $^{e}P < 0.001$, *vs* Model. NC: Normal control group; Model: Ulcerative colitis (UC) model group; si-Ctrl: Transfected with empty vector based on the UC model; si- NCAPD2: Transfected with si-NCAPD2 based on the UC model; si-NCAPD3: Transfected with si-NCAPD3 based on the UC model; si-NCAPD2+si-NCAPD3: Transfected with si-NCAPD2 and si-NCAPD3 based on the UC model; NCAPD2: Non-SMC condensin I complex subunit D2; NCAPD3: Non-SMC condensin II complex subunit D3.

Immunofluorescence results in Figure 4A show that the IKK nuclear volume increased in the Model and si-Ctrl groups compared with that of the NC group; however, with si-NCAPD2 or/and si-NCAPD3 transfection, the IKK nuclear volume was reduced. Figure 4B shows that the NF-κB volume increased in the Model and si-Ctrl groups compared with that of the NC group. However, upon si-NCAPD2 or/and si-NCAPD3 transfection, the NF-κB volume was reduced.

DISCUSSION

UC is a chronic nonspecific inflammatory disease of the rectum and colon that occurs due to intestinal immune abnormalities based on certain hereditary susceptibility, and is affected by environmental factors and enteric microorganisms[10,13]. Previous studies have found that NCAPD2/3 is closely related to the occurrence of various diseases[7,8]. Whether NCAPD2/3 is correlated with the development of UC remains unclear. In this study, the expression levels of NCAPD2 and NCAPD3 in UC intestinal tissues were first detected using ISH. The results showed that the expression levels of NCAPD2 and NCAPD3 were significantly increased in UC intestinal tissues, suggesting that the high expression of NCAPD2/3 might be the key factor leading to UC. Moreover, cell experiments found that the levels of IL-1β, IL-6, and TNF-α were significantly reduced after silencing NCAPD2/3, indicating that this approach can effectively improve the inflammatory reactions induced by UC.

The NF-κB pathway is the primary inflammatory signal transduction pathway, and is involved in the expression and regulation of a variety of inflammatory genes[14]. Under normal circumstances, NF-κB binds to the IκB protein, which normally resides in the cytoplasm in its inactive state[15]. However, under external stimuli (such as proinflammatory cytokines), IκB kinase becomes phosphorylated, resulting in the

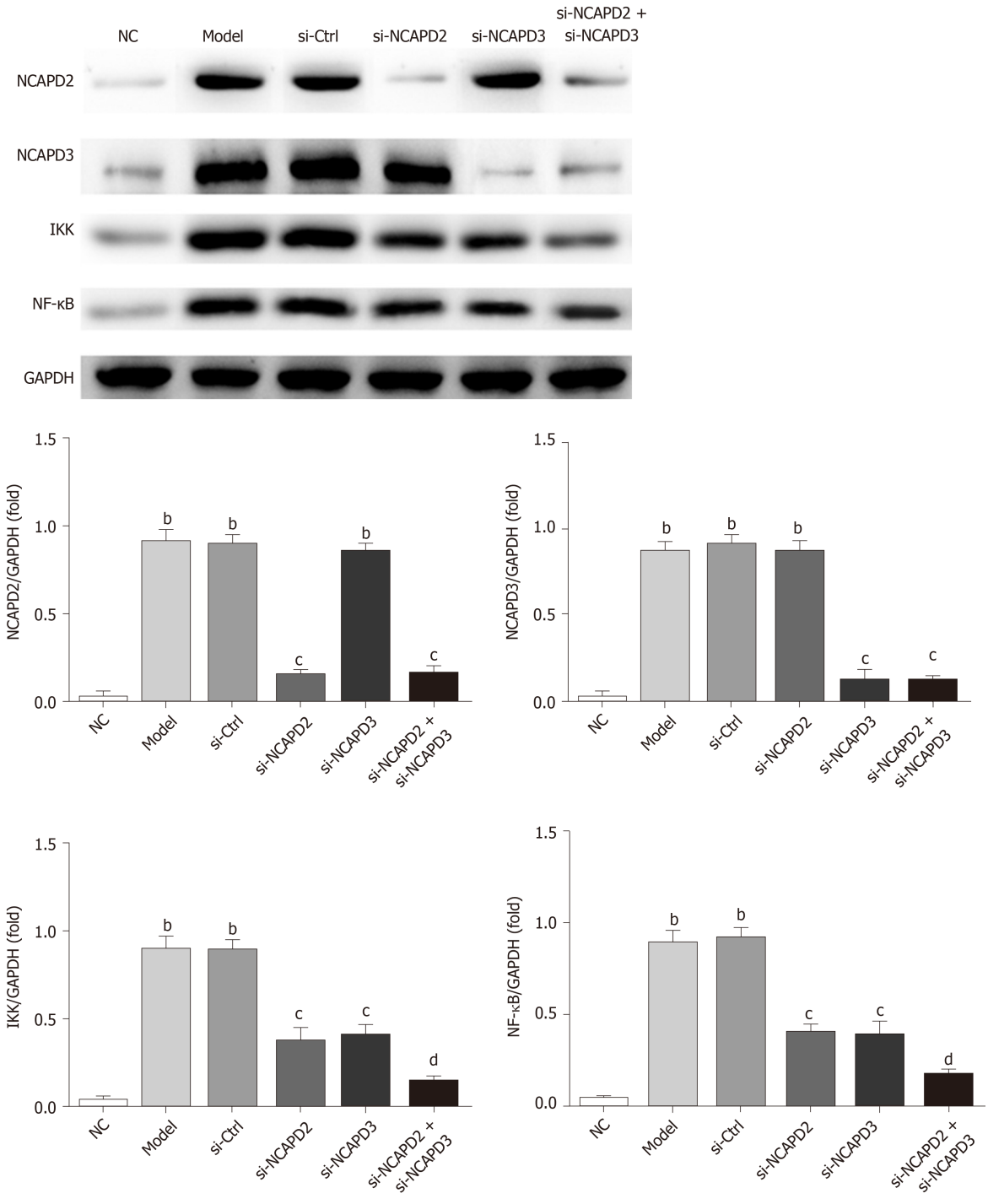

Figure 3 The relative protein expression levels by Western blot assay. $^{b}P < 0.001$ *vs* NC; $^{c}P < 0.01$, $^{d}P < 0.001$ *vs* Model. NC: Normal control group; Model: Ulcerative colitis (UC) model group; si-Ctrl: Transfected with empty vector based on the UC model; si-NCAPD2: Transfected with si-NCAPD2 based on the UC model; si-NCAPD3: Transfected with si-NCAPD3 based on the UC model; si-NCAPD2 + si-NCAPD3: Transfected with si-NCAPD2 and si-NCAPD3 based on the UC model; NCAPD2: Non-SMC condensin I complex subunit D2; NCAPD3: Non-SMC condensin II complex subunit D3.

phosphorylation of IκB, which dissociates from NF-κB and enters the nucleus to regulate the secretion and expression of inflammatory factors[16]. Related studies have confirmed that the activation of IKK/NF-κB is an essential cause of severe inflammation[17-19]. The activation of IKK/NF-κB increases the secretion of the proinflammatory cytokines TNF-α and IL-6, which induces inflammatory reactions[20]. The results of this study revealed that activation of the IKK/NF-κB signaling pathway induced by LPS was effectively inhibited after NCAPD2/3 knockout, which may be one of the important mechanisms for reducing the levels of inflammatory factors (IL-1β, IL-6 and TNF-α).

In summary, we found for the first time that NCAPD2/3 are upregulated in the

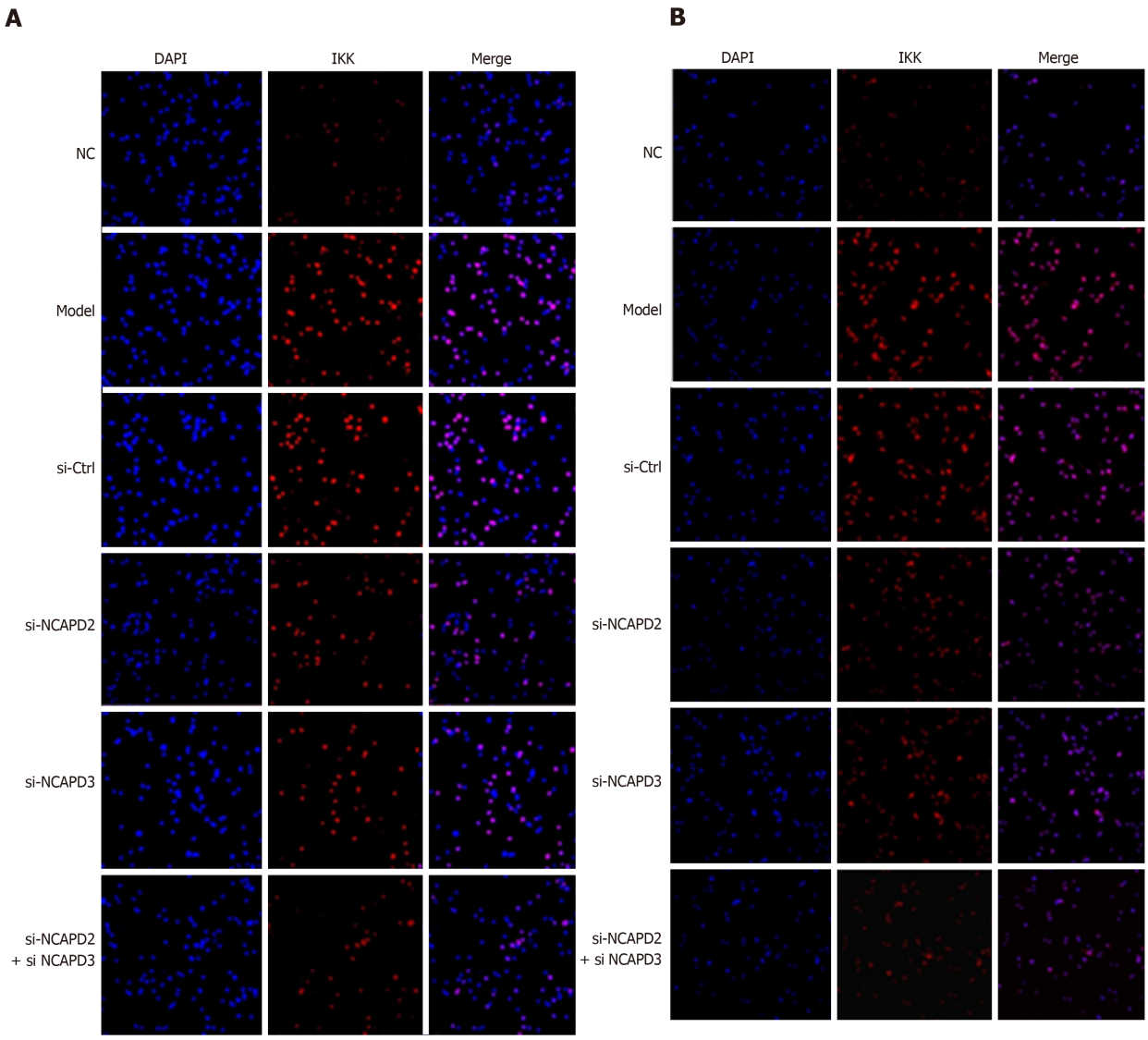

Figure 4 IKK and NF-κB protein expression by immunofluorescence (× 200). NC: Normal Control group; Model: Ulcerative colitis (UC) model group; si-Ctrl: Transfected with empty vector based on the UC model; si-NCAPD2: Transfected with si-NCAPD2 based on the UC model; si-NCAPD3: Transfected with si-NCAPD3 based on the UC model; si-NCAPD2+si-NCAPD3: Transfected with si-NCAPD2 and si-NCAPD3 based on the UC model; NCAPD2: Non-SMC condensin I complex subunit D2; NCAPD3: Non-SMC condensin II complex subunit D3.

intestinal mucosa of patients with active UC, and that this mechanism may involve stimulating the secretion of inflammatory factors IL-1β, IL-6 and TNF-α by activating the IKK/NF-κB pathway. However, given the exploratory and retrospective design of our study, as well as the small clinical sample size, the result of these findings will need to be further validated. Future work will be aimed at deeply investigating the role of NCAPD2/3 in the onset and progression of IBD, including UC and Crohn's disease.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory disease affecting the colon, and the precise molecular mechanisms are undefined. To date, some evidence suggested that non-SMC condensin I complex subunit D2 (NCAPD2) and non-SMC condensin II complex subunit D3 (NCAPD3) play important roles in mitosis and meiosis. Recently, it has been suggested in the literature that subunits of condensing I and condensin II are involved in human cancers, included colorectal cancer. Schuster *et al*[12] has reported that NCAPD3 down-regulates the transcription of genes that encode amino acid transporters (SLC7A5 and SLC3A2) to promote bacterial autophagy by colonic epithelial cells.

Research motivation

To date, there are few studies regarding the correlation of NCAPD2 and NCAPD3 with human diseases, especially in UC. We hypothesize that NCAPD2/3 can also be a potential pathogenic or diagnostic target for ulcerative colitis, and could be used as a new therapeutic target in the future.

Research objectives

In this study, we identified high expression of NCAPD2/3 in the intestinal mucosa of patients with UC. We also analyzed the NCM60 colonic epithelial cell line expressing inducible siRNAs targeting NCAPD2/3, and for the first time, we found that NCAPD2/3 may induce inflammation *via* the IKK/NF-κB pathway in UC. These findings reveal an important role for NCAPD2/3 in UC, thus providing a potential new direction for UC research.

Research methods

We used in situ hybridization (ISH) to measure levels of NCAPD2/3 in intestinal tissue from patients with UC and healthy individuals. *In vitro*, the inflammatory cytokines IKK and NF-κB were evaluated by ELISA, WB and immunofluorescence assay with NCM60 cells expressing small hairpin RNAs against NCAPD2/3.

Research results

In this study, we found that NCAPD2 and NCAPD3 protein expression in intestinal tissue was significantly higher in UC patients than in healthy people. We also found that knockdown of NACPD2/3 in normal colonic epithelial cells (NCM460 cell) resulted in a significant downregulation of IL-1β, IL-6 and TNF-α, possibly by regulating the IKK/NF-κB signaling pathway.

Research conclusions

Levels of NCAPD2/3 proteins are increased in patients with active UC. NCAPD2/3 promote the release of inflammatory cytokines such as IL-1β, IL-6 and TNF-α, which modulate the IKK/NF-κB signaling pathway.

Research perspectives

Our findings indicate a critical role for NCAPD2/3 in the onset and progression of inflammatory bowel disease, as well as strategies to decrease NCAPD2/3 levels that might inhibit inflammation in patients with active UC.

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ORIGINAL ARTICLE

Basic Study

MiR-96-5p inhibition induces cell apoptosis in gastric adenocarcinoma

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Author contributions: Zhou HY designed the research; Chun QW performed the research; Chun QW and Bi EX analyzed the data and wrote the paper.

Institutional review board statement: This study was reviewed and approved by the Jinan Seventh People's Hospital Ethics Committee.

Informed consent statement: All patients in our study provided informed consent.

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

Data sharing statement: No additional data are available.

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Abstract

BACKGROUND

Gastric adenocarcinoma (GAC) mortality rates have remained relatively changed over the past 30 years, and it continues to be one of the leading causes of cancer-related death.

AIM

To search for novel miRNAs related to GAC prognosis and further investigate the effect of miR-96-5p on MGC-803 cells.

METHODS

The miRNA expression profile data of GAC based on The Cancer Genome Atlas were obtained and used to screen differently expressed miRNAs (DEMs) and DEMs related to GAC prognosis. Then, the expression of DEMs related to GAC prognosis was identified in GAC tumor samples and adjacent normal samples by qRT-PCR. The target gene, *ZDHHC5*, of miR-96-5p was predicted using TargetScan, miRTarBase, and miRDB databases and confirmed by luciferase reporter assay. Furthermore, MGC-803 cells were transfected with inhibitor NC, miR-96-5p inhibitor, si-ZDHHC5, or miR-96-5p inhibitor + si-ZDHHC5, and then cell apoptosis was detected by flow cytometry. The expression of ZDHHC5, Bcl-2, and COX-2 was detected using western blotting.

RESULTS

A total of 299 DEMs and 35 DEMs related to GAC prognosis were screened based on The Cancer Genome Atlas. Then compared with adjacent normal samples, the levels of miR-96-5p, miR-222-5p, and miR-652-5p were remarkably increased, while miR-125-5p, miR-145-3p, and miR-379-3p levels were reduced in GAC tumor samples ($P < 0.01$), which were consistent with bioinformatics analysis. Furthermore, ZDHHC5 was defined as a direct target gene of miR-96-5p. miR-96-

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5p inhibition increased the number of apoptotic cells as well as promoted the expression of ZDHHC5, Bcl-2, and COX-2 in MGC-803 cells ($P < 0.01$). After ZDHHC5 inhibition, the number of apoptotic cells and the expression of ZDHHC5, Bcl-2, and COX-2 were reduced. The addition of an miR-96-5p inhibitor partly reversed these effects ($P < 0.01$).

CONCLUSION
Our findings identified six miRNAs related to GAC prognosis and suggested that downregulated miR-96-5p might induce cell apoptosis *via* upregulating ZDHHC5 expression in MGC-803 cells.

Key words: Gastric adenocarcinoma; Differently expressed miRNAs; Prognosis; MicroRNA-96-5p; Cell apoptosis; The Cancer Genome Atlas

Core tip: Gastric adenocarcinoma (GAC) is the most common malignant tumor. It is important to further reveal novel diagnostic and therapeutic methods as well as the underlying molecular mechanism of GAC. This study aimed to search for novel miRNAs related to GAC prognosis. Six miRNAs related to prognosis, including miR-96-5p, miR-125-5p, miR-145-3p, miR-222-5p, miR-379-3p, and miR-652-5p, were identified in GAC samples. Furthermore, downregulated miR-96-5p markedly induced cell apoptosis through targeting ZDHHC5. Current findings provide a potential molecular mechanism of miR-96-5p in GAC.

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INTRODUCTION

Gastric adenocarcinoma (GAC) is the most common malignant tumor originating in the stomach and is counted as one of the top ten common cancers worldwide, with approximately 951000 diagnosed cases and 723000 deaths in 2012[1,2]. Currently, the common and effective therapeutic method is the combination of surgery and adjuvant radiation therapy or chemotherapy, which have improved the 5-year survival rate of GAC[3]. However, delayed diagnosis occurs in most patients with proximal or distant metastasis due to the nontypical symptoms of early GAC, which results in poor treatment and prognosis[4]. Therefore, it is important to further reveal novel diagnostic and therapeutic methods as well as the underlying molecular mechanism of GAC.

It is widely known that a major challenge for the treatment of GAC is poor prognosis, and environmental exposure and gene mutation have been identified to be associated with this outcome[5]. Plenty of evidence indicates that the poor prognosis of GAC is significantly related to many molecular biomarkers, such as microRNAs (miRNA)[6,7]. miRNAs, as endogenous noncoding small-molecule RNAs, widely exist in severe conditions[8]. It is known to function in post-transcriptional regulation of gene expression through binding 3′-untranslated region of their target mRNA, accordingly modulating various key cell biological processes, such as embryonic development, tumor cell proliferation, differentiation, and apoptosis[8,9].

Previous studies have demonstrated that miRNA dysregulation significantly influences the prognosis of gastric cancer patients (*e.g.*, miRNA-203[10], miR-21[11], and miR-25[12]. Imaoka *et al*[10] reported that a low serum miR-203 expression is associated with poor prognosis and may be a noninvasive biomarker for prognosis of gastric cancer patients. Simonian *et al*[11] observed that circulating miR-21 may be considered as a diagnostic and prognostic biomarker in gastric cancer. In addition, Li *et al*[12] revealed that miR-25 is associated with the prognosis of gastric cancer and can induce cell migration and proliferation by targeting transducer of ERBB2.1. Thus, it is essential to search for more novel miRNAs related to GAC prognosis, which may contribute to the development of GAC diagnosis.

In the current study, the miRNA expression profile data of GAC based on The

Cancer Genome Atlas (TCGA) were analyzed to screen differently expressed miRNAs (DEMs) and DEMs related to GAC prognosis. Furthermore, DEMs were identified in clinical samples, and the mechanism of DEM was investigated *in vitro*. According to this, we aimed to search for new therapeutic targets for GAC and provide some useful insights in improving the prognosis of GAC patients.

MATERIALS AND METHODS

Data extraction and DEM screening

The miRNA expression profile data (level 3, processed and standardized data) and the corresponding clinical information of GAC were downloaded from TCGA (https://portal.gdc.cancer.gov/) on February 11, 2019 based on the platform of Illumina HiSeq 2000 RNA Sequencing platform. A total of 452 samples were obtained from this dataset, including 410 GAC tumor samples and 42 normal control samples. The edgeR package in R was utilized to screen DEMs between GAC samples and normal samples. The thresholds were defined as false discovery rate < 0.05 and |log fold change| > 1. Meanwhile, volcano plots and heat maps were generated based on the obtained DEMs.

DEMs screening related to prognosis

The overall survival time was individually extracted from clinical information. Then, combined with the overall survival times and the expression levels of DEMs, DEMs related to prognosis were screened using KMsurv package of R, with the threshold of log-rank $P < 0.05$.

Clinical validation sample collection

This study obtained ethical approval from the ethics committee of Jinan Seventh People's Hospital, and the study was performed according to the Helsinki Declaration. A total of 20 paired tumor tissues and adjacent normal tissues (distance of 3-4 cm from the tumor tissue) were collected from GAC patients who underwent surgery in Jinan Seventh People's Hospital between September 2018 to September 2019. The specimens were confirmed by hematoxylin eosin staining and stored in RNA later. In addition, 5 mL peripheral blood was obtained from these 20 GAC patients. Meanwhile, the same amount of peripheral blood was extracted from 20 paired healthy subjects. Written informed consent from all participants was obtained, and the clinical information, including age, weight, gender, distant metastasis, lymph node metastasis, depth of invasion, and TNM stage are shown in Table 1.

Predicting target genes of DEMs

Target genes of DEMs related to prognosis were predicted using the three online analysis databases, including miRDB, miRTarBase, and TargetScan. Overlapping target genes among the three tools were selected to make the bioinformatic analysis more reliable. Then, the intersection of the predicting target genes among the three databases was obtained using a Venn diagram online tool, and the target genes that overlapped in the three databases were considered as a potential target gene of DEM.

Cell culture and transfection

Human gastric carcinoma cell line MGC-803 was obtained from Shanghai Obio Technology Co., Ltd. The cells were maintained in Dulbecco's Modified Eagle Media (DMEM, Gibco, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, United States). The construction of ZDHHC5 silence vector (si-ZDHHC5) was performed by GenePharma (Shanghai, China). The miR-96-5p inhibitor and inhibitor NC were purchased from Thermo (Waltham, MA, United States). MGC-803 cells were inoculated in six-well plates for 24 h with approximately 5×10^5 cells in each well, and then inhibitor NC, miR-96-5p inhibitor, si-ZDHHC5, or miR-96-5p inhibitor + si-ZDHHC5 was transfected into MGC-803 cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) per the manufacturer's instructions. Meanwhile, MGC-803 cells without transfection served as the control group. Cells were harvested after 48 h of transfection to perform follow-up experiments.

Real-time quantitative polymerase chain reaction

Total RNA from tissues and peripheral blood was obtained by Trizol (Invitrogen) and RNeasy Plus Mini Kit (Qiagen, Valencia, CA, United States) per the manufacturer's instructions. The concentration of RNA was detected using NanoDrop ND-2000 (Invitrogen). To generate cDNA, Mir-X™ miRNA FirstStrand Synthesis Kit (Takara, Dalian, China) was used. The quantitative polymerase chain reaction (qPCR) was

Table 1 Distribution of characteristics in gastric adenocarcinoma patients and healthy subjects

Variables	Patients, *n* = 20		Controls, *n* = 20		*P* value[1]
	n	%	*n*	%	
Age in yr, mean ± SD	62.1 ± 5.2		60.1 ± 10.2		0.73
Weight in kg, mean ± SD	64.0 ± 7.1		69.1 ± 6.6		0.88
Gender					
Male	13	65.0	1	55.0	0.64
Female	7	35.0	9	45.0	
Depth of invasion					
T1/T2	7	35.0			
T3/T4	13	65.0			
Lymph node metastasis					
N0	6	30.0			
N1/N2/N3	14	70.0			
Distant metastasis					
M0	8	40.0			
M1	12	60.0			
TNM stage					
I/II	7	35.0			
III/IV	13	65.0			

Data are presented as *n* (%) unless otherwise indicated.
[1]Independent-samples *t*-test and two-sided χ^2 test for selected variables distributions between cases and controls. SD: Standard deviation.

carried out using the SYBR Premix Ex*Taq* TM II (Takara) by ABI 7900 qRT-PCR System (Applied Biosystems, Foster City, CA, United States). The primer sequences are listed in Table 2. U6 and glyceraldehyde-phosphate dehydrogenase were used as the internal control of measuring miR-19a and *ADIPOR2* expression. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Luciferase reporter assay

The target gene of miR-96-5p was verified using the luciferase assay. The 3′-untranslated region of ZDHHC5 was cloned into a pGL3-basic vector, named as Luc-ZDHHC5. Luc-ZDHHC5 and phRL-TK plasmid were co-transfected with miR-96-5p mimic, miR-96-5p NC (negative control), or siZDHHC5 (positive control) (synthesized by Biosyntech, Suzhou, China) into 293T cells. After 48 h of transfection, the relative luciferase activity was measured by the Dual-Glo Luciferase Assay System (Promega) in accordance to the manufacturer's introductions. Renilla luciferase activity was used to normalize luciferase activity.

Western blotting

Total proteins were isolated by RIPA Lysis Buffer (Beyotime, Shanghai, China). Proteins concentrations were tested by bicinchoninic acid kit (Beyotime). The protein sample was separated on SDS-PAGE gel, transferred to polyvinylidene fluoride membranes, and followed by the blockage with 5% nonfat milk for 1 h. Next, the membranes were probed with primary antibodies of ZDHHC5 (1:1000, Proteintech), Bcl-2 (1:1000, Abcam), COX-2 (1:1000, Abcam), and GAPDH (1:1000, Beyotime) overnight at 4 °C. Then, membranes were incubated with secondary antibody (1:1000, Beyotime) for 2 h in a dark room at room temperature. GAPDH was used as the control protein. Enhanced chemiluminescence Plus reagent (Beyotime) was used to image blots. The band quantification was performed using Image J software.

Flow cytometry assay

Annexin V-FITC Apoptosis Detection kit was used to evaluate cell apoptosis. MGC-803 cells were grown in 6-well plates for 24 h and then transfected with inhibitor NC, miR-96-5p inhibitor, si-ZDHHC5, or miR-96-5p inhibitor + si-ZDHHC5 for 48 h. Next, cells were digested with trypsin and washed with PBS, followed by resuspending in 1 × Binding Buffer, and stained with propidium iodide and FITC-Annexin V for 15 min at 25 °C in the dark. Cells were finally detected using a flow cytometer (Beckman Coulter, Fullerton, CA, United States).

Table 2 Primers used for the quantitative real-time polymerase chain reaction

Gene	Primer sequence
miR-96-5p	F:5′-TCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCAAAAA-3′
	R: 5′- ACACTCCAGCTGGGTTTGGCACTAGCACATT-3′
miR-125a-5p	F: 5′- CCCTGAGACCCTTTAACCT-3′
	R: 5′- GTCCAGTTTTTTTTTTTTTTTCACAG-3′
miR-145-3p	F: 5′- GGTCCAGTTTTCCCAGGA-3′
	R: 5′- CCAGTTTTTTTTTTTTTTTAGGGATTC-3′
miR-222-5p	F: 5′- GCTCAGTAGCCAGTGTAGA-3′
	R: 5′- GTCCAGTTTTTTTTTTTTTTTAGGATCT-3′
miR-379-3p	F: 5′- GCAGTGGTAGACTATGGAAC-3′
	R: 5′- GGTCCAGTTTTTTTTTTTTTTCCT-3′
miR-652-5p	F: 5′- CCTAGGAGAGGGTGCCA-3′
	R: 5′- GTCCAGTTTTTTTTTTTTTTTGAATGG-3′
miR-708-3p	F: 5′- GCAACTAGACTGTGAGCTTC-3′
	R: 5′- GGTCCAGTTTTTTTTTTTTTTTCTAGA-3′
GAPDH	F: 5′-AGAAGGCTGGGGCTCATTTG-3′
	R: 5′-AGGGGCCATCCACAGTCTTC-3′
U6	F: 5′-AGGGGCCATCCACAGTCTTC-3′
	R: 5′-AACGCTTCACGAATTTGCGT-3′

GAPDH: Glyceraldehyde-phosphate dehydrogenase.

Statistical analysis

Statistical analysis was conducted using SPSS Statistics software 22.0 (Chicago, IL, United States). Continuous variables were expressed as mean ± standard deviation and analyzed by independent-samples *t* test. Categorical variables were expressed as percentages and assessed by two-sided chi-square test. The differences of multiple groups were performed by one-way ANOVA following with post-hoc of Dunnett *t* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

DEMs between GAC sample and normal sample based on TCGA

Based on the selective criteria, a total of 299 DEMs were identified between GAC and normal control samples, including 225 upregulated and 74 downregulated miRNAs. As shown in Figure 1A and 1B, volcano plots and heat maps were conducted for these 299 DEMs.

DEMs related to prognosis based on TCGA

Based on these 299 DEMs, the relationships between patient overall survival and miRNA expression were evaluated, and the results showed that 35 DEMs were significantly related to the prognosis of GAC patients ($P < 0.05$). Among these DEMs, seven miRNAs had a higher association with GAC prognosis ($P < 0.01$), including miR-96-5p ($P = 8.049 \times 10^{-3}$), miR-125-5p ($P = 9.638 \times 10^{-4}$), miR-145-3p ($P = 6.002 \times 10^{-3}$), miR-222-5p ($P = 1.812 \times 10^{-3}$), miR-379-3p ($P = 5.032 \times 10^{-3}$), miR-652-5p ($P = 3.145 \times 10^{-3}$), and miR-708-3p ($P = 7.984 \times 10^{-3}$) (Figure 2).

DEMs identification in clinical samples

A total of 20 GAC patients and 20 healthy subjects were included in this study. No significant difference was found in age, weight, and gender between GAC patients and healthy subjects (Table 1). Based on the above survival analysis, six miRNAs were selected for identification in GAC tumor samples and adjacent normal samples. qRT-PCR revealed that compared with adjacent normal samples, the levels of miR-96-5p, miR-222-5p, and miR-652-5p were remarkably increased, while miR-125-5p, miR-145-3p, and miR-379-3p levels were obviously reduced in GAC tumor samples ($P < 0.01$, Figure 3A), which was consistent with bioinformatics analysis results by TCGA. Moreover, miR-96-5p level was detected in the blood of GAC patients and healthy subjects, but no significant difference was found (Figure 3B).

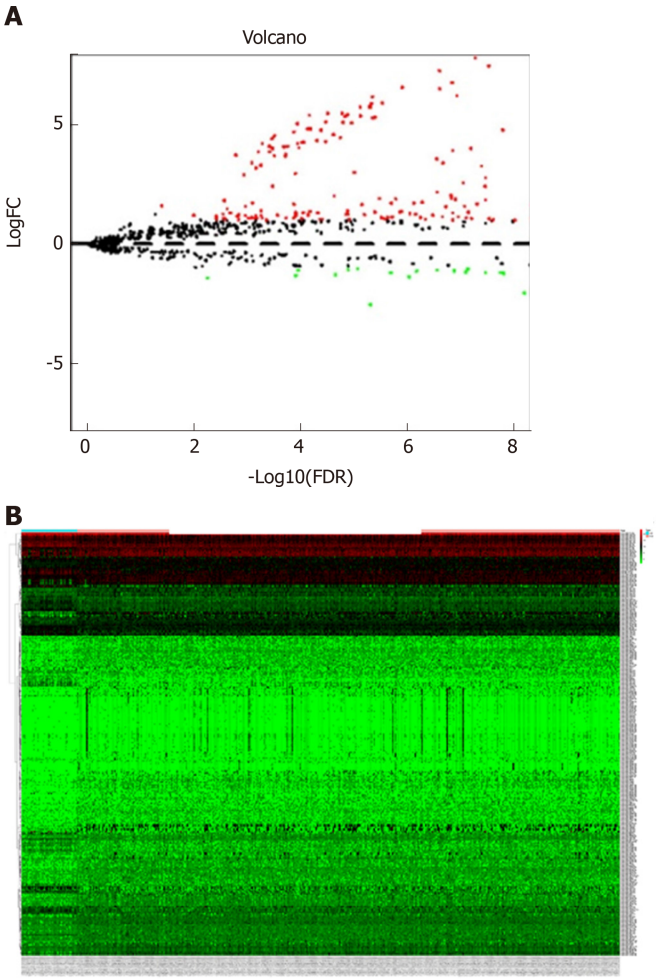


Figure 1 Differentially expressed miRNAs between gastric adenocarcinoma sample and normal sample. A: Volcano plot for differentially expressed miRNA (DEM) expression. Dark dots represent upregulated miRNAs, whereas lighter dots represent downregulated miRNAs; B: Hierarchical gene clustering analysis of DEMs represented by a heat map. Dark represents upregulated miRNAs, whereas lighter represents downregulated miRNAs.

Target gene prediction and identification of miR-96-5p

Considering miR-96-5p had the highest association with GAC prognosis, the function of miR-96-5p was investigated in the following experiments. The results found that a total of 39 overlapped target genes existed in TargetScan, miRTarBase, and miRDB databases (Figure 4A). Based on this bioinformatics analysis, *ZDHHC5* was considered as a potential target gene of miR-96-5p (Figure 4B). Luciferase receptor assay showed that the relative luciferase activity was reduced after co-transfection with miR-96-5p mimic or siZDHHC5 compared with co-transfection with miR-96-5p NC (Figure 4C), which suggested *ZDHHC5* was a direct target gene of miR-96-5p.

Effect of miR-96-5p on apoptosis in MGC-803 cells

To further investigate the effects of miR-96-5p on GAC, the miR-96-5p inhibitor was used to inhibit miR-96-5p in MGC-803 cells. Flow cytometry assay showed that the number of apoptotic cells increased in MGC-803 cells transfected with the miR-96-5p inhibitor, while inhibition of *ZDHHC5* decreased cell apoptosis compared with cells with NC. Notably, co-transfection of the miR-96-5p inhibitor and si-ZDHHC5 partly reversed the effect of inhibiting *ZDHHC5* on cell apoptosis ($P < 0.01$, Figure 5A). In addition, western blotting revealed that compared with MGC-803 cells without treatment, miR-96-5p inhibition promoted the expression of ZDHHC5, Bcl-2, and COX-2 (apoptosis proteins) in MGC-803 cells ($P < 0.01$, Figure 5B). However, inhibiting *ZDHHC5* decreased the expression of ZDHHC5, Bcl-2, and COX-2. The addition of the miR-96-5p inhibitor increased the expression of these three proteins ($P < 0.01$, Figure 5B).

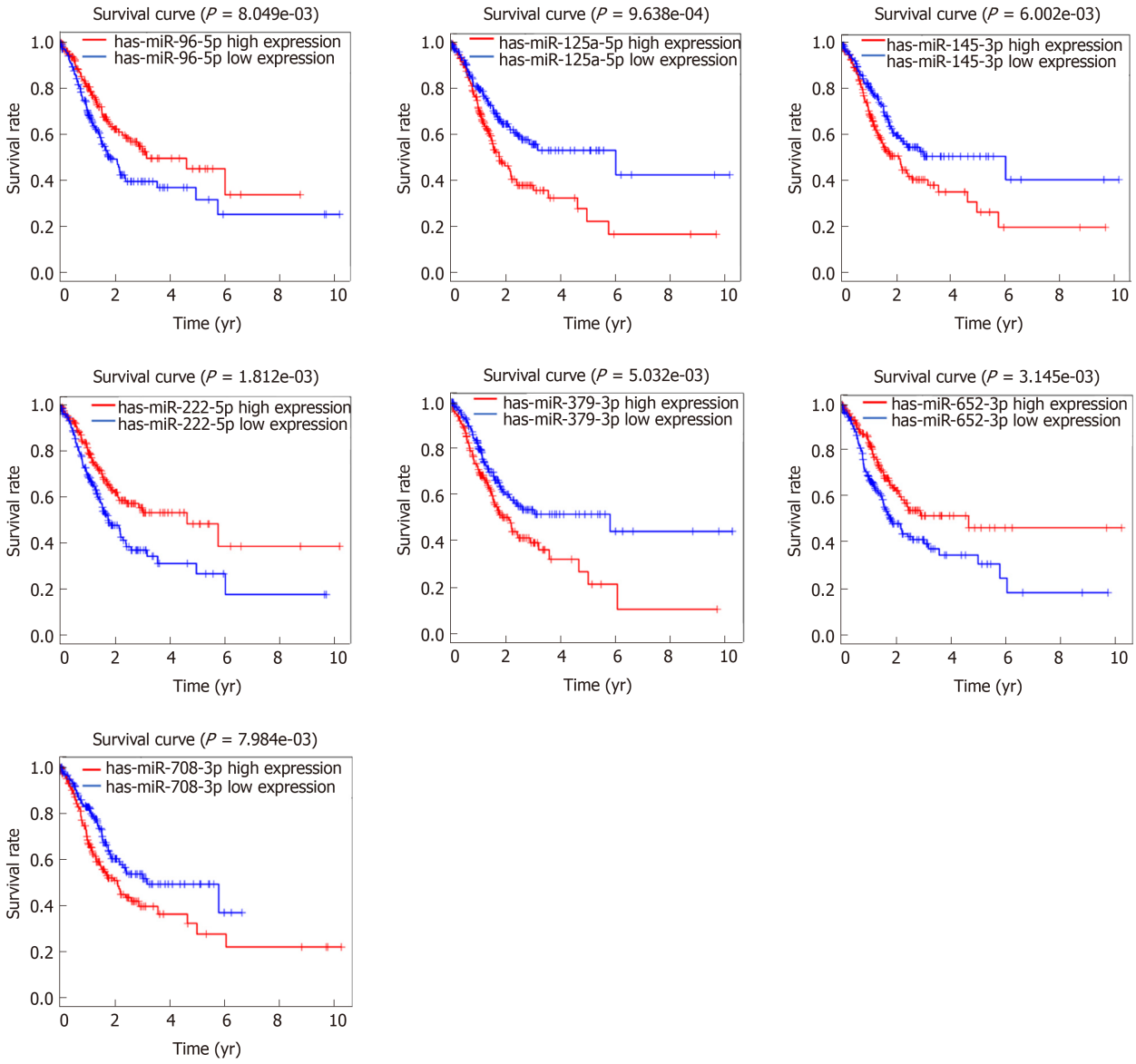


Figure 2 Association of differentially expressed miRNAs with overall survival of gastric adenocarcinoma. Dark lines represent high expression, and lighter lines represent low expression.

DISCUSSION

In the present study, a total of 299 DEMs and 35 DEMs related to GAC prognosis were screened based on the miRNA expression profile data from TCGA. Then, six miRNAs were selected for identification in GAC tumor samples and adjacent normal samples. The results were consistent with bioinformatics analysis. Furthermore, miR-96-5p was considered as an important biomarker and investigated in the *in vitro* experiments. Our results revealed that *ZDHHC5* was a direct target gene of miR-96-5p, and miR-96-5p inhibition increased the expression of Bcl-2 and COX-2.

Six miRNAs were identified in this study, and the results showed that the levels of miR-96-5p, miR-222-5p, and miR-652-5p were overexpressed, while miR-125-5p, miR-145-3p, and miR-379-3p levels were downregulated in GAC sample. Several studies have demonstrated that miR-96-5p is overexpressed in various cancers, including colorectal cancer[13], pancreatic carcinoma[14], prostate cancer[15], hepatocellular carcinoma[16], and breast cancer[17], and it is an oncogene by promoting cell proliferation. miR-652-5p was reported to be associated with non-small cell lung cancer[18], esophageal adenocarcinoma[19], and breast cancer[20], while the mechanism of miR-652-5p was unknown. Current studies of miR-222-5p are focused on the role of angiogenesis in endothelium[21,22], and few studies investigated the effect of miR-222-5p in cancers. miR-125-5p was identified as a tumor suppressor in glioblastoma[23], cervical cancer[24], and renal cell carcinoma[25], and it was involved in proliferation, migration, and apoptosis. Many have investigated the role of miR-145-3p in cancers,

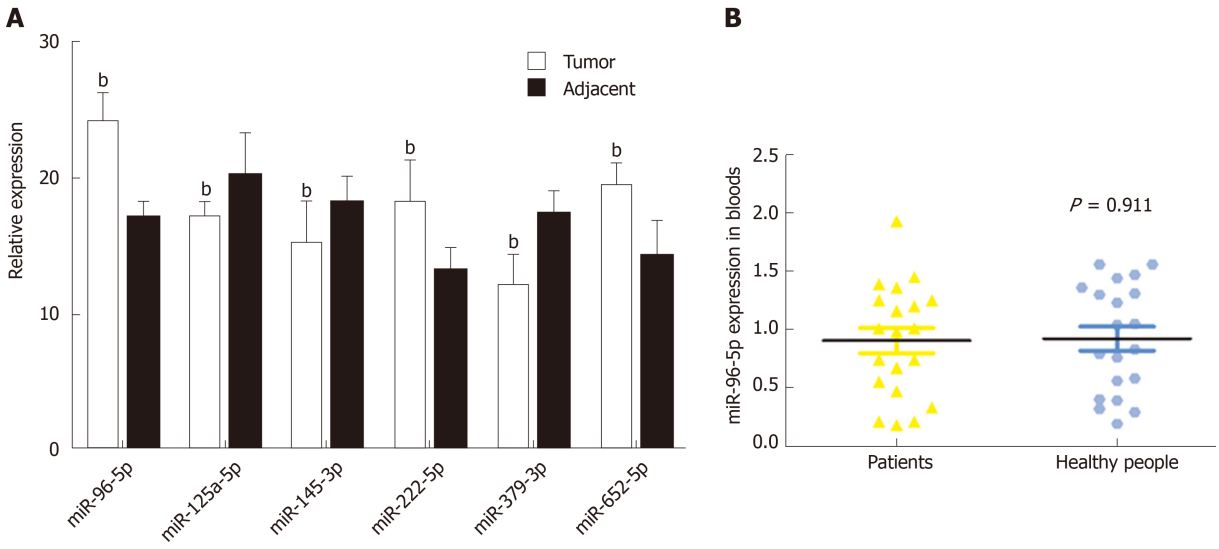


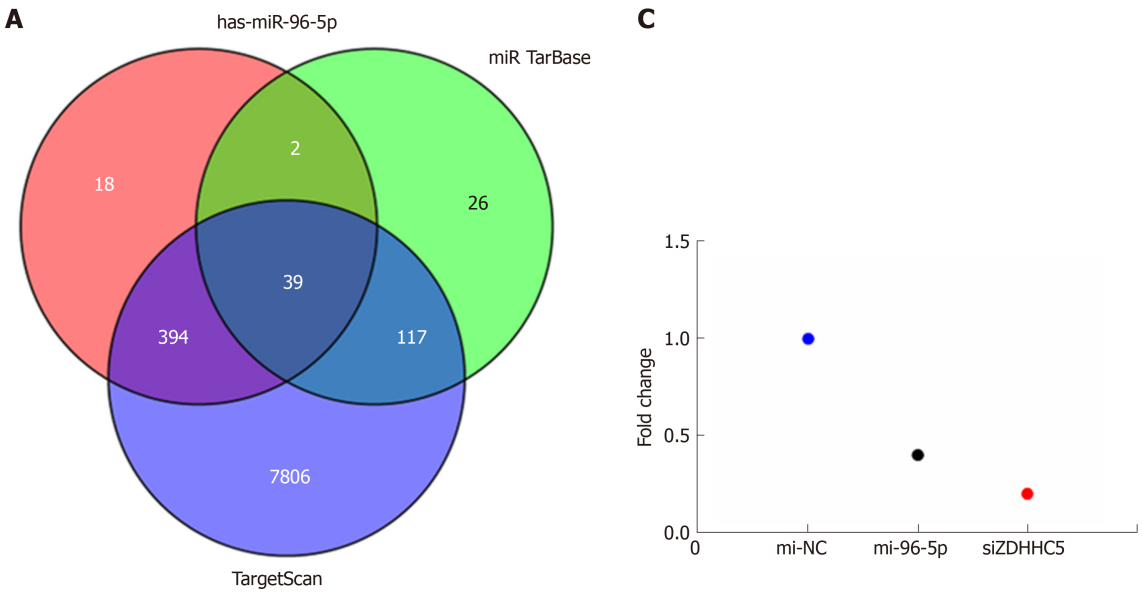
Figure 3 The identification of differentially expressed miRNA levels in gastric adenocarcinoma patients and healthy subjects. A: The levels of miR-96-5p, miR-222-5p, miR-652-5p, miR-125-5p, miR-145-3p, and miR-379-3p in tumor samples and adjacent normal samples by qRT-PCR; B: The miR-96-5p level in the blood of gastric adenocarcinoma patients and healthy subjects by qRT-PCR ($^bP < 0.01$). Values are mean ± SD.

such as bladder cancer[26], lung squamous cell carcinoma[27], gallbladder cancer[28], and head and neck squamous cell carcinoma[29]. It is also considered a tumor suppressor. Few studies have investigated the role of miRNA-379-3p; only one recent study reported that miRNA-379-5p exerted an antitumor effect by regulating tumor invasion and metastasis in hepatocellular carcinoma[30]. Unfortunately, the effects of these miRNAs on GAC have not been reported. Therefore, it is essential to further reveal the mechanism and prognostic significance of these miRNAs in GAC.

Due to the highest association of miR-96-5p with GAC prognosis, the effects of miR-96-5p on MGC-803 cells were investigated in this study. Notably, a previous study has shown that miR-96-5p exerts an inhibiting role in cell proliferation and migration by downregulation of FoxQ1 in gastric cancer cells[31]. Contradictorily, a recent study has demonstrated that miR-96-5p exerts a promoting effect on cell progression by directly targeting FOXO3 in gastric cancer. Consistent with this recent study[32], this study found that the miR-96-5p inhibitor induced cell apoptosis in MGC-803 cells.

It is generally acknowledged that miRNAs develop biological functions by impeding translation of target mRNAs. In agreement with the bioinformatics prediction, our study revealed that *ZDHHC5* was identified as a target gene of miR-96-5p. *ZDHHC5*, encoding zinc finger DHHC-type containing 5, is one member of the family of ZDHHC proteins and was identified as a putative palmitoyl S-acyltransferases[33]. It has been suggested that S-palmitoylation is closely associated with cancer development, and ZDHHC enzymes are the key enzymes responsible for palmitoylation[34].

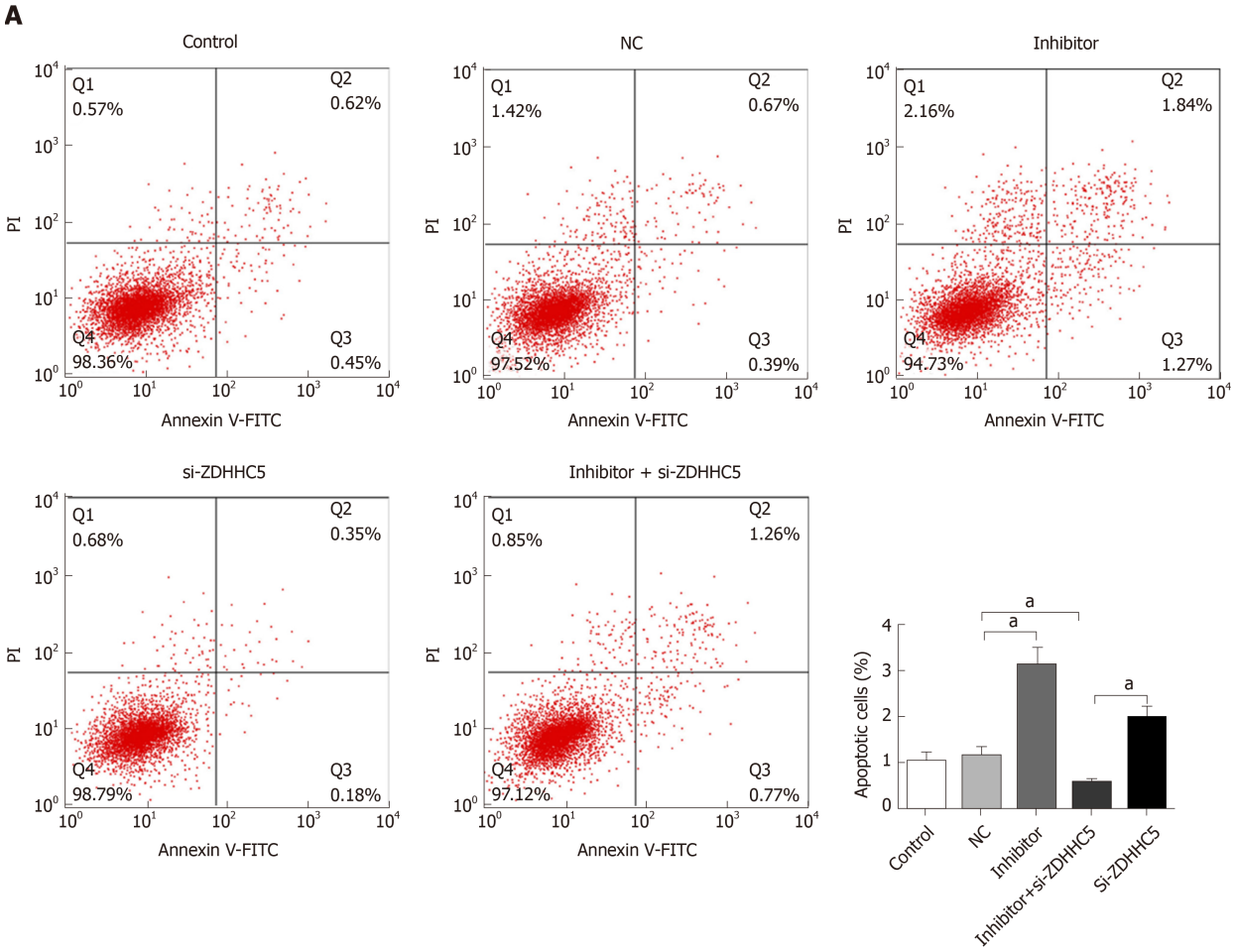
Individual ZDHHC enzymes exert different effects on various cancers, either as tumor suppressors or oncoproteins[34]. A previous study documented that high expression of ZDHHC5 is associated with a poor prognosis in glioma[35]. In addition, the report of Tian *et al*[36] has suggested that DHHC5 knockdown can dramatically inhibit cell proliferation and invasion in non–small cell lung cancer. The present study revealed that miR-96-5p inhibition increased the number of apoptotic cells as well as promoted the expression of ZDHHC5, Bcl-2, and COX-2 in MGC-803 cells, while inhibiting *ZDHHC5* decreased cell apoptosis. Co-transfection of the miR-96-5p inhibitor and si-ZDHHC5 partly reversed the effect of *ZDHHC5* inhibition on cell apoptosis. These results indicated that miR-96-5p inhibition induced cell apoptosis by upregulating ZDHHC5 expression. This result was inconsistent with previous studies, which may be due to ZDHHC5 having different functions in different cancer types[37].



B

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved Branch length	P_{CT}
Position 927-933 of ZDHHC5 3′ UTR has-miR-96-5p	5`…AUAUUUUUCAUCACAUGCCAAAA… 3`ACUCACGAACGAUCCACGGUUC	7mer-A1	-0.17	85	-0.01	4.934	0.73
Position 927-933 of ZDHHC5 3′ UTR has-miR-1271-5p	5`…AUAUUUUUCAUCACAUGCCAAAA… 3`UCGUUUUUACACGAUCACGGUUU…	7mer-A1	-0.13	81	-0.01	4.934	0.73

Figure 4 Target gene prediction and identification of miR-96-5p. A: The intersection of the predicting target genes of miR-96-5p among the TargetScan, miRTarBase and miRDB databases using a Venn diagram; B: Prediction of the binding site of miR-96-5p to *zinc finger DHHC-type palmitoyltransferase 5* (*ZDHHC5*) by bioinformatics; C: Target regulation of miR-96-5p to *ZDHHC5* observed by the luciferase reporter system. ZDHHC5: Zinc finger DHHC-type palmitoyltransferase 5.



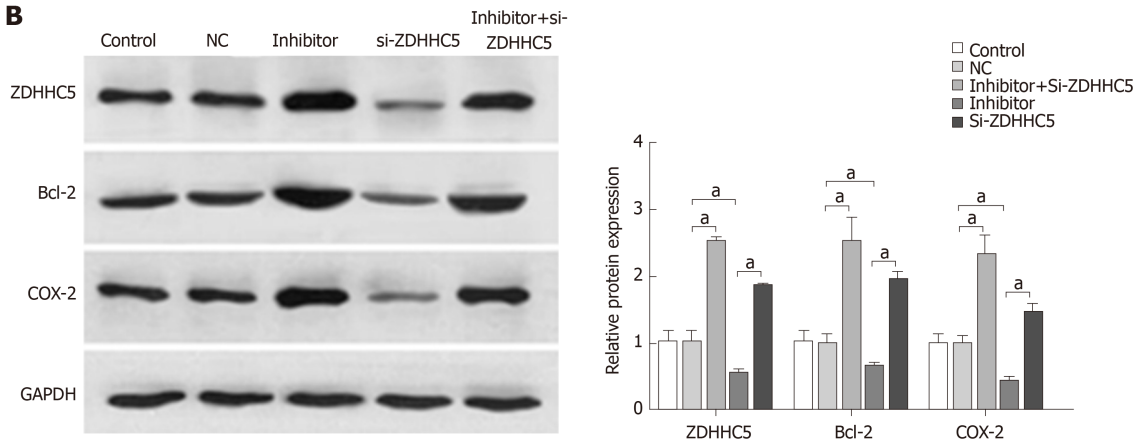


Figure 5 miR-96-5p inhibition might induce cell apoptosis in MGC-803 cells. A: The number of apoptotic cells after transfection with inhibitor NC, miR-96-5p inhibitor, si-zinc finger DHHC-type palmitoyltransferase 5 (ZDHHC5), or miR-96-5p inhibitor + si-ZDHHC5 in MGC-803 cells by flow cytometry analysis; B: The expression of ZDHHC5, Bcl-2, and COX2 after cells were treated with inhibitor NC, miR-96-5p inhibitor, si-ZDHHC5, or miR-96-5p inhibitor + si-ZDHHC5 in MGC-803 cells by western blotting ($^aP < 0.01$). Values are mean ± SD. ZDHHC5: Zinc finger DHHC-type palmitoyltransferase 5.

ARTICLE HIGHLIGHTS

Research background

Gastric adenocarcinoma (GAC) is one of the leading causes of cancer-related death. However, delayed diagnosis is found in most patients with proximal or distal metastasis due to the nontypical symptoms of early GAC, which results in poor treatment and prognosis. Therefore, it is important to further reveal novel diagnostic and therapeutic methods as well as the underlying molecular mechanism of GAC.

Research motivation

Plenty of evidence indicates that the poor prognosis of GAC is significantly related to many

molecular biomarkers, such as microRNA (miRNA). Previous studies have demonstrated that miRNA dysregulation significantly influences the prognosis of gastric cancer patients (*e.g.*, miRNA-203, miR-21, and miR-25). Thus, it is essential to search for novel miRNAs related to GAC prognosis, which may contribute to the development of GAC diagnosis.

Research objectives
This study aimed to search for new miRNA therapeutic targets for GAC and investigate the mechanism of differentially expressed miRNA (DEM) *in vitro*, which might provide some useful insights in improving the prognosis of GAC patients.

Research methods
First, the miRNA expression profile data of GAC based on The Cancer Genome Atlas were obtained and used to screen DEMs and DEMs related to GAC prognosis by bioinformatics methods. Then, the expression of DEMs related to GAC prognosis was identified in GAC tumor samples and adjacent normal samples by qRT-PCR. *ZDHHC5*, a target gene of miR-96-5p, was predicted and confirmed by the luciferase reporter assay. Furthermore, MGC-803 cells were transfected with inhibitor NC, miR-96-5p inhibitor, si-ZDHHC5, or miR-96-5p inhibitor + si-ZDHHC5. Cell apoptosis was detected by flow cytometry. The expression of ZDHHC5, Bcl-2, and COX-2 was detected using western blotting.

Research results
A total of 299 DEMs and 35 DEMs related to GAC prognosis were screened based on the miRNA expression profile data from The Cancer Genome Atlas. Six miRNAs, including miR-96-5p, miR-222-5p, miR-652-5p, miR-125-5p, miR-145-3p, and miR-379-3p, were selected for identification in GAC tumor samples and adjacent normal samples. The results were consistent with bioinformatics analysis. Furthermore, miR-96-5p was considered as an important biomarker and investigated in *in vitro* experiments. Our results revealed that *ZDHHC5* was a direct target gene of miR-96-5p, and miR-96-5p inhibition induced cell apoptosis and increased the expression of Bcl-2 and COX-2.

Research conclusions
In conclusion, this work identified six miRNAs related to GAC prognosis, including miR-96-5p, miR-125-5p, miR-145-3p, miR-222-5p, miR-379-3p, and miR-652-5p. Furthermore, downregulated miR-96-5p markedly induced cell apoptosis through targeting *ZDHHC5*.

Research perspectives
Current findings provide a potential molecular mechanism of miR-96-5p in GAC. However, further studies are needed to investigate the mechanism and prognostic significance of these miRNAs in GAC.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Trends in treatment and overall survival among patients with proximal esophageal cancer

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Abstract

BACKGROUND

The management of proximal esophageal cancer differs from that of tumors located in the mid and lower part of the esophagus due to the close vicinity of vital structures. Non-surgical treatment options like radiotherapy and definitive chemoradiation (CRT) have been implemented. The trends in (non-)surgical treatment and its impact on overall survival (OS) in patients with proximal esophageal cancer are unclear, related to its rare disease status. To optimize treatment strategies and counseling of patients with proximal esophageal cancer, it is therefore essential to gain more insight through real-life studies.

AIM

To establish trends in treatment and OS in patients with proximal esophageal cancer.

METHODS

In this population-based study, patients with proximal esophageal cancer diagnosed between 1989 and 2014 were identified in the Netherlands Cancer Registry. The proximal esophagus consists of the cervical esophagus and the upper thoracic section, extending to 24 cm from the incisors. Trends in radiotherapy, chemotherapy, and surgery, and OS were assessed. Analyses were stratified by presence of distant metastasis. Multivariable Cox proportional hazards regression analyses was performed to assess the effect of period of diagnosis on OS, adjusted for patient, tumor, and treatment characteristics.

RESULTS

In total, 2783 patients were included. Over the study period, the use of radiotherapy, resection, and CRT in non-metastatic disease changed from 53%, 23%, and 1% in 1989-1994 to 21%, 9%, and 49% in 2010-2014, respectively. In metastatic disease, the use of chemotherapy and radiotherapy increased over time. Median OS of the total population increased from 7.3 mo [95% confidence interval (CI): 6.4-8.1] in 1989-1994 to 9.5 mo (95%CI: 8.1-10.8) in 2010-2014 (logrank $P < 0.001$). In non-metastatic disease, 5-year OS rates improved from 5% (95%CI: 3%-7%) in 1989-1994 to 13% (95%CI: 9%-17%) in 2010-2014 (logrank $P < 0.001$). Multivariable regression analysis demonstrated a significant treatment effect over time on survival. In metastatic disease, median OS was 3.8 mo (95%CI: 2.5-5.1) in 1989-1994, and 5.1 mo (95%CI: 4.3-5.9) in 2010-2014 (logrank $P = 0.26$).

CONCLUSION

OS significantly improved in non-metastatic proximal esophageal cancer, likely to be associated with an increased use of CRT. Patterns in metastatic disease did not change significantly over time.

Key words: Esophagus; Esophageal cancer; Proximal; Cervical; Upper thoracic; Trends; Treatment; Survival; Outcome

Core tip: Proximal esophageal cancer is a rare disease, accounting for only 10% of all esophageal cancer cases. Limited data on treatment and survival in this rare tumor have been published, restricting patient counseling. The present investigation is the largest population-based cohort study evaluating trends in treatment and survival in proximal esophageal cancer. This study represents daily clinical practice, showing improvement in overall survival in patients with non-metastatic proximal esophageal cancer, with a shift to non-surgical treatment.

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INTRODUCTION

Esophageal cancer is the seventh most common cancer worldwide[1]. Although the absolute number of deaths has decreased, esophageal cancer is still the sixth leading cause of cancer-related mortality globally[1]. Surgical treatment of patients with esophageal cancer, and in particular treatment of cancer located in the proximal part of the esophagus, is challenging because of the close proximity to vital structures. The proximal part of the esophagus consists of the cervical and the upper thoracic segment. Proximal esophageal cancer is relatively uncommon, accounting for 10% of all esophageal cancer cases[2].

The management of proximal esophageal cancer differs from that of tumors located in the mid and lower part of the esophagus. Patients with proximal esophageal cancer often present with locally advanced disease, for which potentially curative surgery would require extensive mutilating resections, with a high risk of major complications and a significant impact on patients quality of life. To prolong survival and improve quality of life, non-surgical treatment options like radiotherapy and definitive chemoradiation (CRT) have been explored since the 1990s, following promising treatment results of cancers in the thoracic esophagus, hypopharynx, and non-small-cell lung cancer[3-6]. In a meta-analysis in 2006, Wong *et al*. showed that the addition of chemotherapy to radiotherapy for the definitive treatment of esophageal cancer significantly increased response and overall survival (OS) rates[7].

Therefore, definitive CRT is recommended as treatment modality for patients with non-metastatic proximal esophageal cancer[8,9]. However, only four of the 19 studies in the aforementioned meta-analysis incorporated patients with proximal esophageal cancers, limiting the extrapolation of these findings to the proximal esophagus.

Separate OS rates for patients with proximal esophageal cancer are largely lacking from clinical trials, due to exclusion of this subpopulation or related to its rare disease status. To optimize treatment strategies and counseling of patients with proximal esophageal cancer, it is therefore essential to gain more insight in patient characteristics, provided therapies and OS through real-life studies.

The aim of this population-based cohort study was to establish the trends in treatment and OS in patients diagnosed with non-metastatic or metastatic proximal esophageal cancer in a nationwide registry between 1989 and 2014.

MATERIALS AND METHODS

Patients

All patients with a tumor located in the cervical or upper thoracic esophagus diagnosed between 1989 and 2014 were identified in the Netherlands Cancer Registry (NCR). The NCR is a population-based cancer registry of all residents of the Netherlands. The NCR is linked to the national automated pathological archive, which leads to the automatic inclusion of all newly diagnosed malignancies in the Netherlands. Additional data sources linked to the NCR are the national hospital discharge register and registers of radiotherapy institutions. Information on vital status was obtained through annual linkage with the Municipal Administrative Database, in which all deceased or emigrated individuals in the Netherlands are registered. This study was approved by the Privacy Review Board of the NCR and the need for a separate approval from an ethics committee in the Netherlands was waived.

Definitions

Topography and histology were coded according to the International Classification of Diseases for Oncology (ICD-O)[10]. ICD-O histology codes were used to classify tumors as squamous cell carcinoma (SCC), adenocarcinoma, and other origin. Cancers of the proximal esophagus can be subdivided in cancers originating in the cervical esophagus (CEC, ICD-O C15.0), commencing at the lower border of the cricoid

cartilage and ending at the thoracic inlet, approximately 18 cm from the incisors, and cancers in the upper thoracic section (UTEC, ICD-O C15.3), extending from the thoracic inlet to the level of the tracheal bifurcation, which is approximately 24 cm from the incisors[11].

Tumor staging was registered according to the Union for International Cancer Control TNM classification that was valid at the time of diagnosis. As the classification of tumor stage (cT) was reasonably comparable from the TNM-4 to -6, but changed with the introduction of the 7th edition in 2010, we converted all tumor and lymph node stages according to TNM-6th edition. Patients with a cM1a tumor according to TNM-6th edition, defined as cervical lymph node involvement, were categorized as having a positive lymph node status (cN+). Patients with unknown metastatic status (cMx) were included in the non-metastatic group.

All treatments for the primary disease stage were registered. Treatment categories included resection, neoadjuvant treatment and resection, radiotherapy, chemotherapy, radiotherapy and chemotherapy, other treatment, and no (anti-cancer) treatment. Resection included patients who received a surgical resection or an endoscopic excision (n = 20). The group of "neoadjuvant and resection" comprised patients who underwent a resection, preceded by radiotherapy, chemotherapy or with concurrent CRT. The group "radiotherapy and chemotherapy" included patients who were treated with sequential or concurrent radiotherapy and chemotherapy, without any resection. Other treatments were not otherwise specified (palliative) treatments. "Other treatment" and "no (anti-cancer) treatment" were summarized as "no localized treatment". Type of surgical treatment and details on chemotherapy or radiotherapy were not collected by the data clerks of the NCR.

Five-year periods of diagnosis were defined: 1989-1994, 1995-1999, 2000-2004, 2005-2009, and 2010-2014.

Statistical analysis

OS was calculated by period of diagnosis using the Kaplan-Meier method and a comparison between groups was made using the log-rank test. OS was defined as the time from diagnosis to death from any cause, censored at last follow-up date or until February 1, 2017. The median follow-up time was calculated using the reverse Kaplan Meier method (death censored). Multivariable Cox proportional hazards regression analyses were performed to assess the effect of period of diagnosis on OS, adjusted for age, histological type, tumor location, cT category, cN category, and treatment modality. Variance inflation factors were calculated to assess the degree of multicollinearity among the independent variables in the Cox proportional hazard model. Analyses were stratified by the presence of metastatic disease (cM0 *vs* cM1), tumor location (CEC *vs* UTEC), and histological type (SCC *vs* adenocarcinoma). As the interaction analysis did not show any difference in OS between tumor location, *i.e.*, cervical or upper thoracic site, and histology, results are presented by presence or absence of metastatic disease.

The statistical review of the study was performed by two senior epidemiologists.

RESULTS

Study population

We identified 2783 patients diagnosed with proximal esophageal cancer in the Netherlands between 1989 and 2014 (Table 1). The median follow-up time of all patients was 103 mo [95% confidence interval (CI): 91-117 mo]. Fifty-six percent of patients were male, and 47% were between 60 and 74 years old at the time of diagnosis. In total, 81% of cancers were SCC. Two percent of the patients were diagnosed with clinical stage 1, 20% with stage 2, 28% with stage 3, 21% with stage 4, and 29% with unknown stage disease. The number of patients with unknown stage disease decreased over time. In 2010-2014, 27% of patients had been diagnosed with another malignancy prior to the diagnosis of proximal esophageal cancer (data not shown).

Trends in treatment in patients with proximal esophageal cancer

In patients with non-metastatic disease, the proportion of patients treated with CRT alone increased from 1% in 1989-1994 to 49% in 2010-2014 (Figure 1A). Resection without neoadjuvant treatment was performed in 17% of patients in 1989-1994 and in 2% of patients in 2010-2014. The proportion of patients treated with neoadjuvant therapy and resection was relatively constant over time, varying between 3% and 7%. The proportion of patients with non-metastatic proximal esophageal cancer that did not undergo any form of treatment varied between 15% and 22%, without a clear

Table 1 Patient and tumor charactistics by time period of diagnosis, *n* (%)

Charactistics	Total (*n* = 2783)	1989-1994 (*n* = 484)	1995-1999 (*n* = 499)	2000-2004 (*n* = 552)	2005-2009 (*n* = 583)	2010-2014 (*n* = 665)
Sex						
Male	1562 (56)	259 (54)	263 (53)	308 (56)	344 (59)	388 (58)
Female	1221 (44)	225 (46)	236 (47)	244 (44)	239 (41)	277 (42)
Age (yr)						
< 60	725 (26)	140 (29)	148 (30)	178 (32)	128 (22)	131 (20)
60-74	1304 (47)	194 (40)	219 (44)	223 (40)	301 (52)	367 (55)
≥ 75	754 (27)	150 (31)	132 (26)	151 (27)	154 (26)	167 (25)
Histology						
SCC	2248 (81)	382 (79)	390(78)	440 (80)	480 (82)	556 (84)
Adenocarcino-ma	320 (11)	62 (13)	63 (13)	70 (13)	61 (10)	64 (10)
Other	215 (8)	40 (8)	46 (9)	42 (8)	42 (7)	45 (7)
Tumor location						
CEC	648 (23)	138 (29)	138 (28)	154 (28)	126 (22)	92 (14)
UTEC	2135 (77)	346 (71)	361 (72)	398 (72)	457 (78)	573 (86)
cT classification						
cT1	81 (3)	17 (4)	16 (3)	12 (2)	16 (3)	20 (3)
cT2	236 (8)	12 (2)	16 (3)	36 (7)	48 (8)	124 (19)
cT3	447 (16)	36 (7)	39 (8)	79 (14)	109 (19)	184 (28)
cT4	665 (24)	115 (24)	123 (25)	161 (29)	147 (25)	119 (18)
cTx	1354 (49)	304 (63)	305 (61)	264 (48)	263 (45)	218 (33)
cN classification						
cN0	892 (32)	172 (36)	173 (35)	189 (34)	157 (27)	201 (30)
cN+	1193 (43)	119 (25)	158 (32)	208 (38)	313 (54)	395 (59)
cNx	698 (25)	193 (40)	168 (34)	155 (28)	113 (19)	69 (10)
cM classification						
cM0	1752 (63)	311 (64)	314 (63)	316 (57)	344 (59)	467 (70)
cM1	589 (21)	79 (16)	88 (18)	96 (17)	135 (23)	191 (29)
cMx	442 (16)	94 (19)	97 (19)	140 (25)	104 (18)	7 (1)
TNM stage						
1	64 (2)	14 (3)	14 (3)	9 (2)	14 (2)	13 (2)
2	565 (20)	80 (17)	72 (14)	100 (18)	125 (22)	188 (28)
3	763 (27)	102 (21)	126 (25)	173 (31)	174 (30)	188 (28)
4	589 (21)	79 (16)	88 (18)	96 (17)	135 (23)	191 (29)
Unknown	802 (29)	209 (43)	199 (40)	174 (32)	135 (23)	85 (13)

Percentages may not add up to 100 percent because of rounding. SCC: Squamous cell carcinoma; CEC: Cervical esophageal cancer; UTEC: Upper thoracic esophageal cancer.

trend over time.

For patients with metastatic disease, only minor variations in treatment were observed (Figure 1B). Fourty-four percent of patients were treated with radiotherapy alone in 1989-1994, which slightly decreased to 37% in 2010-2014. Over time, multimodal treatment of chemotherapy and radiotherapy, concurrent or sequential, was administered more frequently: In 3% of patients in 1989-1994 and 23% of patients in 2010-2014. Chemotherapy alone was given to 7%-12% of patients in all time periods. The proportion of patients diagnosed with metastatic proximal esophageal cancer who did not undergo any form of anti-cancer treatment decreased from 33% in 1989-2004 to 24% in 2010-2014.

Trends in survival in patients with proximal esophageal cancer

The median OS of the total population of patients with proximal esophageal cancer was 8.0 mo (95%CI: 7.6-8.5 mo). Median OS increased over the study period, from 7.3 mo (95%CI: 6.4-8.1 mo) in 1989-1994, to 9.5 mo (95%CI: 8.1-10.8 mo) in 2010-2014 (logrank $P < 0.001$) (Figure 2). In patients with non-metastatic proximal esophageal cancer, 1- and 5-year OS rates improved from 30% (95%CI: 26%-34%) and 5% (95%CI:

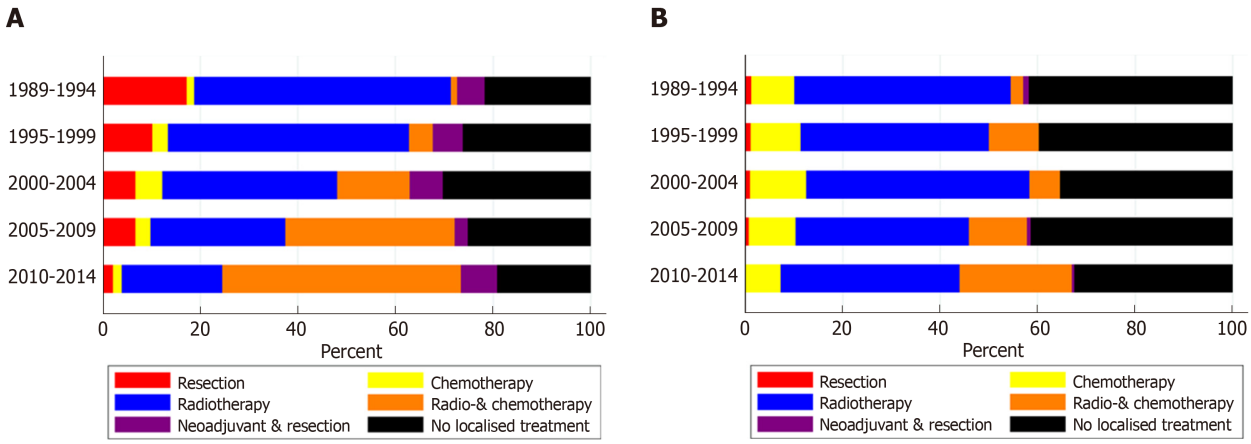


Figure 1 Treatment of patients with proximal esophageal cancer in the Netherlands between 1989 and 2014. A: Patients with non-metastatic proximal esophageal cancer; B: Patients with metastatic proximal esophageal cancer.

3%-7%) in 1989-1994, to 44% (95%CI: 40%-48%) and 13% (95%CI: 9%-17%) in 2010-2014, respectively (logrank $P < 0.001$) (Figure 3A). Median OS of patients with non-metastatic proximal esophageal cancer was 8.0 mo (95%CI: 7.0-8.9 mo) in 1989-1994 and 13.3 mo (95%CI: 11.1-15.5 mo) in 2010-2014. Patients with stage 1 disease showed the most favorable outcome with a 1- and 5-year OS rate of 70% (95%CI: 57%-80%) and 22% (95%CI 13%-34%), compared with 50% (95%CI: 46%-54%) and 15% (95%CI: 12%-18%) in stage 2, and 35% (95%CI: 32%-38%) and 10% (95%CI: 8%-13%) in stage 3 disease, respectively (logrank $P < 0.001$) (Supplementary Figure 1).

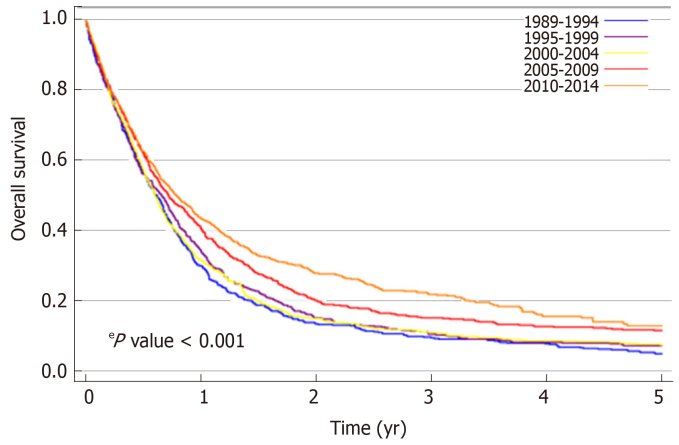
In patients with non-metastatic proximal esophageal cancer, univariable analysis showed that period of diagnosis, age, histological type, cT, cN, and treatment were all associated with OS (Table 2). OS was similar for patients diagnosed with CEC or UTEC. Multivariable Cox regression analysis adjusted for age, histological type, tumor location, cT, and cN demonstrated an OS benefit for patients diagnosed in 2005-2009 [Hazard ratio (HR) = 0.77, $P < 0.001$] or 2010-2014 (HR = 0.72, $P < 0.001$) when compared with patients diagnosed in 1989-1994. However, the time period effect disapeared after additional inclusion of treatment modality in the multivariable model. All treatment modalities had a statistically significant effect on OS compared with no localized treatment ($P < 0.001$). Patients with non-metastatic proximal esophageal cancer treated with surgery with or without neoadjuvant therapy or treated with definitive CRT showed 5-year OS rates of 31% (95%CI: 23%-40%), 21% (95%CI: 16%-28%), and 22% (95%CI: 19%-26%), respectively (logrank $P = 0.32$) (Supplementary Figure 2).

In patients with metastatic disease, OS did not change significantly over time (logrank $P = 0.26$) (Figure 3B). Median OS was 3.8 mo (95%CI: 2.5-5.1 mo) in 1989-1994 and 5.1 mo (95%CI: 4.3-5.9 mo) in 2010-2014. One-year OS rate was 12% (95%CI: 6%-20%) in 1989-1994 and 23% (95%CI: 17%-29%) in 2010-2014.

DISCUSSION

In the Netherlands, median OS of patients with proximal esophageal cancer significantly increased by approximately two mo between 1989 and 2014. In patients with non-metastatic proximal esophageal cancer, 5-year OS almost tripled to 13% in 2010-2014, although the absolute longterm outcome remains poor. Multivariable analysis showed that improvements in treatment over time might have led to this survival benefit. The improvement is likely to be attributable to the implementation of CRT in the late nineties, accounting for almost 50% of treatment choices in non-metastatic proximal esophageal cancer nowadays. The proportion of patients who did not receive any anti-cancer treatment remained remarkably high, being one in five patients with non-metastatic and one in four patients with metastatic proximal esophageal cancer, which may be a reflection of the poor performance status of these patients.

We observed that in the patients with non-metastatic proximal esophageal cancer ($n = 2194$), the median OS improved from 8 mo in 1989-1994 to 13 mo in 2010-2014, with comparable OS between CEC and UTEC. Considering OS in patients with metastatic disease did not improve significantly over time, stage migration was not expected to be a major contributor to the improved survival in the non-metastatic



Number at risk	0	1	2	3	4	5
1989-1994	475	141	63	45	36	23
1995-1999	498	172	75	52	40	36
2000-2004	550	174	82	60	47	40
2005-2009	579	238	116	88	73	67
2010-2014	664	290	154	84	38	17

Figure 2 Overall survival by 5-year period of diagnosis of patients with proximal esophageal cancer in the Netherlands between 1989 and 2014, irrespective of stage at diagnosis.

group. A Surveillance, Epidemiology, and End Results (SEER) data-based study in 362 patients with non-metastatic CEC diagnosed between 1998 and 2008 showed a longer median OS, *i.e.*, 14 mo[12]. The shorter median survival observed in our study may partly be explained by the inclusion of patients with a history of previous malignancies, whereas the SEER data-based study excluded these patients. In addition, we included patients with unknown metastatic status in the group of patients with non-metastatic disease, which could have lead to an underestimation of the OS in the non-metastatic patient group.

Our study showed a reduction of surgical approaches from 23% in the earliest time period to 10% in the most recent period. The aforementioned SEER population-based study showed similar results, where only 11% of patients with cervical esophageal cancer underwent surgery and 79% radiotherapy (chemotherapy data were not available)[12]. These findings confirm a different approach in the management of proximal esophageal cancer in specific as compared with cancers from all sites of the esophagus. In the latter group the proportion of patients treated with surgery remained relatively stable over time, from 25% between 1989 and 2004, to 29% between 2010 and 2014[2].

Considering bias by indication, we hypothesized that patients with resectable tumors, undergoing surgery, might show a superior outcome when compared with CRT. However, in the current population-based study, we observed a comparable OS in patients treated with surgery *vs* those treated with definitive CRT which is consistent with a recent observational study in 148 patients with cervical esophageal cancer[13]. The current study showed that period effect in the multivariable model dissapeared after including treatment modality. These findings suggest that improvements in the (non-surgical) treatment had a substantial effect on the observed improvement in OS. However progress in OS may also have partly occurred due to advancements in the management of non-cancer related high mortality disorders, *e.g.*, cardiovascular disease[14]. Figures from Statistics Netherlands show that the remaining life expectancy for, for example, an average 65 year old person was 17 years in 1989 and 20 years in 2014[15]. Whether this increase in life expectancy is also seen in the high-risk population presented in our study is unknown.

In patients with metastatic proximal esophageal cancer, we did not observe any significant improvements in OS over time. These findings are in contrast to previous population-based studies, observing an increased survival over the years in the total group of patients with metastatic esophageal cancer patients, including 10% of cancers originating from the proximal esophagus[16,17]. This difference in the trend in OS may be explained by the more prominent increased use of systemic therapy in metastatic adenocarcinomas[2], which are more common in the distal part of the esophagus[18]. For example, in patients with HER2 amplified adenocarcinomas of the distal esophagus, HER2 directed therapies have led to a survival benefit[19]. In

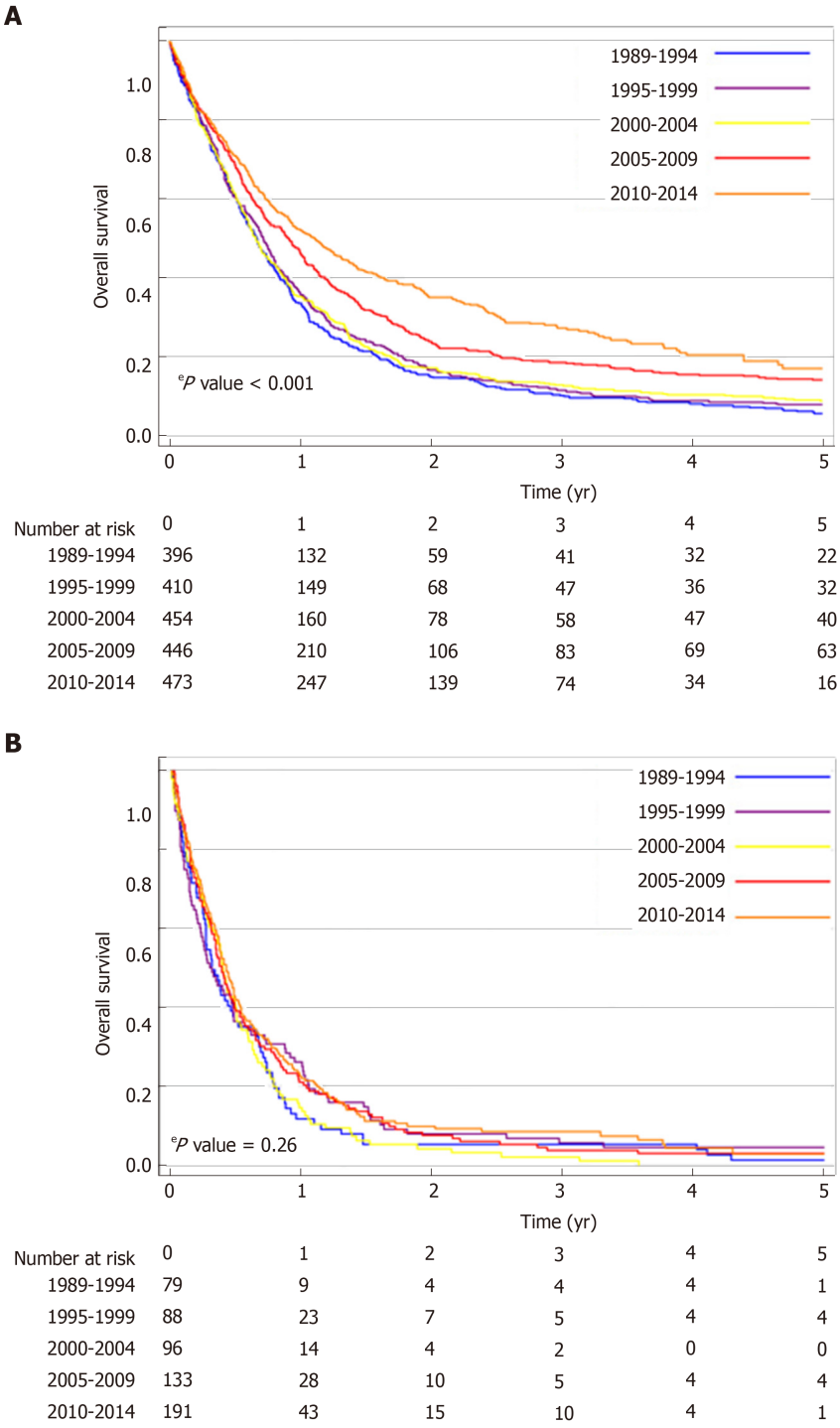


Figure 3 Overall survival by 5-year period of diagnosis of patients with proximal esophageal cancer in the Netherlands between 1989 and 2014. A: Patients with non-metastatic proximal esophageal cancer; B: Patients with metastatic proximal esophageal cancer.

metastatic SCC, palliative systemic therapy is scarcely applied[2]. A recent meta-analysis, however, showed that systemic therapy in patients with metastatic SCC improved OS and quality of life, and is considered standard of care[20]. The outcomes of patients with metastatic SCC is expected to improve in the coming decades, because the pace of development of cancer immunotherapies is accelerating. Recent studies show clinical evidence of efficacy of immune checkpoint inhibitors in SCC of the esophagus[21,22], and are expected to be approved for implementation in clinical practice.

Furthermore, since proximal esophageal cancer is extremely rare, development of high-volume expert centers is challenging. Centralization of surgery in esophageal cancer has led to an increased survival in resectable esophageal cancer[23]. A recent

Table 2 Univariable and multivariable hazard ratios for overall survival of patients diagnosed with non-metastatic proximal esophageal cancer (*n* = 2194)

Charactistics	*n*	Univariable analysis		Multivariable analysis		Multivariable analysis[1]	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Period							
1989-1994	405	Ref.		Ref.		Ref.	
1995-1999	411	0.92 (0.80-1.05)	0.21	0.91 (0.79-1.05)	0.18	0.85 (0.74-0.98)	0.03
2000-2004	456	0.92 (0.81-1.06)	0.24	0.97 (0.85-1.12)	0.71	0.94 (0.82-1.08)	0.39
2005-2009	448	0.73 (0.63-0.83)	< 0.001	0.77 (0.67-0.89)	< 0.001	0.88 (0.76-1.02)	0.09
2010-2014	474	0.59 (0.51-0.68)	< 0.001	0.72 (0.62-0.85)	< 0.001	0.94 (0.79-1.10)	0.43
Age							
< 60 yr	562	Ref.		Ref.		Ref.	
60-74 yr	1002	1.06 (0.95-1.18)	0.30	1.11 (0.99-1.23)	0.08	0.97 (0.86-1.08)	0.53
≥ 75 yr	630	1.50 (1.33-1.69)	< 0.001	1.51 (1.34-1.71)	< 0.001	1.00 (0.87-1.14)	0.95
Histology							
SCC	1797	Ref.		Ref.		Ref.	
AC	242	1.07 (0.93-1.23)	0.37	0.97 (0.84-1.12)	0.64	0.88 (0.76-1.02)	0.09
Other	155	1.47 (1.24-1.74)	< 0.001	1.22 (1.03-1.44)	0.02	1.11 (0.93-1.31)	0.25
Tumor location							
UTEC	1672	Ref.		Ref.		Ref.	
CEC	522	0.97 (0.88-1.08)	0.59	0.89 (0.80-0.98)	0.02	0.95 (0.86-1.06)	0.37
cT category							
cT1-3	642	Ref.		Ref.		Ref.	
cT4	506	2.03 (1.79-2.30)	< 0.001	1.93 (1.69-2.19)	< 0.001	1.62 (1.42-1.85)	< 0.001
cTx	1046	1.75 (1.57-1.94)	< 0.001	1.50 (1.33-1.69)	< 0.001	1.25 (1.11-1.41)	< 0.001
cN category							
cN0	825	Ref.		Ref.		Ref.	
cN+	811	1.29 (1.16-1.43)	< 0.001	1.44 (1.29-1.60)	< 0.001	1.35 (1.21-1.50)	< 0.001
cNx	558	2.06 (1.84-2.30)	< 0.001	1.78 (1.59-2.00)	< 0.001	1.37 (1.22-1.55)	< 0.001
Treatment							
No localized treatment	538	Ref.				Ref.	
Resection	183	0.19 (0.16-0.22)	< 0.001			0.22 (0.18-0.26)	< 0.001
Neoadjuvant and resection	126	0.15 (0.12-0.18)	< 0.001			0.17 (0.13-0.21)	< 0.001
Radio- and chemotherapy	480	0.17 (0.14-0.19)	< 0.001			0.19 (0.16-0.22)	< 0.001
Chemotherapy	67	0.38 (0.29-0.49)	< 0.001			0.39 (0.30-0.50)	< 0.001
Radiotherapy	800	0.38 (0.34-0.42)	< 0.001			0.40 (0.36-0.46)	< 0.001

[1]Additionally adjusted for treatment category. SCC: Squamous cell carcinoma; AC: Adenocarcinoma; CEC: Cervical esophageal cancer; UTEC: Upper thoracic esophageal cancer; HR: Hazard ratio; CI: Confidence interval.

Dutch study showed that center volume of palliative systemic therapy for metastatic esophagogastric cancer was associated with improved survival, suggesting a volume-outcome relationship[24]. Giving the low incidence rate and the challenging performance status of these patients, this could be a plea for centralization of care for patients with proximal esophageal cancer.

The retrospective nature of this study is inherent with some limitations mainly attributable to the availability of information. Coding of the tumor was being performed on the basis of topography, extracted from the medical records depending on input of physicians and interpretation of administrators, posing a risk of misclassification. The NCR does not include information on treatment techniques, schedules, and its related toxicities, causing interpretation adversity. Furthermore, data regarding risk factors, *e.g.*, smoking behaviour and alcohol consumption, comorbidity, performance status, and disease specific cause of death were not available, resulting in a risk of residual confounding. However, our multivariable model showed that the period effect almost completely dissapeared after including treatment modalities to the multivariable model, implicating that there are no major confounders missing.

The strength of our study is that it is a large population-based cohort. This nationwide cohort of patients with proximal esophageal cancer in the Netherlands represents daily clinical practice, reflecting real-life treatment and survival. Moreover, the follow-up period can be considered long, given the relatively short survival time of patients with proximal esophageal cancer.

In conclusion, this nationwide study in patients with proximal esophageal cancer showed an increasing use of definitive CRT over the study period, with improved survival in non-metastatic disease, although long-term result is still rather poor.

ARTICLE HIGHLIGHTS

Research background

Proximal esophageal cancer is a rare disease, accounting for only 10% of all esophageal cancers. Nearby vital structures are involved in almost all proximal esophageal cancers at diagnosis, and as such surgical treatment is mutilating with major implications for quality of life of patients. Definitive chemoradiation (CRT) is an alternative treatment option, but survival data are scarce, restricting patient counseling.

Research motivation

To optimize treatment strategies and counseling of patients with proximal esophageal cancer, it is therefore essential to gain more insight in patient characteristics, provided therapies and outcome through real-life studies.

Research objectives

The aim of this population-based cohort study was to establish the trends in treatment and overall survival (OS) in patients diagnosed with non-metastatic or metastatic proximal esophageal cancer in a nationwide registry between 1989 and 2014.

Research methods

All patients with a tumor located in the cervical or upper thoracic esophagus diagnosed between 1989 and 2014 were identified in the Netherlands Cancer Registry (NCR). The NCR is a population-based cancer registry of all residents of the Netherlands. Trends in radiotherapy, chemotherapy, and surgery, and OS were assessed. Analyses were stratified by presence of distant metastasis. Multivariable Cox proportional hazards regression analyses was performed to assess the effect f period of diagnosis on OS, adjusted for adjust for patient, tumor, and treatment characteristics.

Research results

Median OS of patients with proximal esophageal cancer significantly increased by approximately two mo between 1989 and 2014. In patients with non-metastatic proximal esophageal cancer, 5-year OS almost tripled to 13% in 2010-2014, although the absolute longterm outcome remains poor. Multivariable analysis showed that improvements in treatment over time have led to this survival benefit. The improvement is likely to be attributable to the implementation of CRT in the late nineties, accounting for almost 50% of treatment choices in non-metastatic proximal esophageal cancer nowadays, as shown in the current study. In metastatic disease, median OS did not change significantly between 1989 and 2014.

Research conclusions

Surgical treatment for proximal esophageal cancer has been substituted by definitive CRT in the more recent years, and was likely to be associated with significant survival improvement of patients with non-metastatic proximal esophageal cancer. (Long-term) survival data of patients with (non-)metastatic proximal esophageal cancer are provided from a large national database, representing daily clinical practice.

Research perspectives

Our findings give insights in real-life survival of patients with proximal esophageal cancer, providing crucial support for patient counseling. Future research should focuss on outcome between different CRT regimens, to optimize non-surgical treatment.

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ORIGINAL ARTICLE

Retrospective Study

Influence of bile contamination for patients who undergo pancreaticoduodenectomy after biliary drainage

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Abstract

BACKGROUND

The influence of bile contamination on the infectious complications of patients undergoing pancreaticoduodenectomy (PD) has not been thoroughly evaluated.

AIM

To evaluate the effect of preoperative biliary drainage and bile contamination on the outcomes of patients who undergo PD.

METHODS

The database of 4101 patients who underwent PD was reviewed. Preoperative biliary drainage was performed in 1964 patients (47.9%), and bile contamination was confirmed in 606 patients (14.8%).

RESULTS

The incidence of postoperative infectious complications was 37.9% in patients with preoperative biliary drainage and 42.4% in patients with biliary contamination, respectively. Patients with extrahepatic bile duct carcinoma, ampulla of Vater carcinoma, and pancreatic carcinoma had a high frequency of preoperative biliary drainage (82.9%, 54.6%, and 50.8%) and bile contamination (34.3%, 26.2%, and 20.2%). Bile contamination was associated with postoperative pancreatic fistula (POPF) Grade B/C, wound infection, and catheter infection. A multivariate logistic regression analysis revealed that biliary contamination (odds ratio 1.33, $P = 0.027$) was the independent risk factor for POPF Grade B/C. The three most commonly cultured microorganisms from bile (*Enterococcus, Klebsiella, and Enterobacter)* were identical to those isolated from organ spaces.

CONCLUSION

In patients undergoing PD, bile contamination is related to postoperative infectious complication including POPF Grade B/C. The management of biliary contamination should be standardised for patients who require preoperative biliary drainage for PD, as the main microorganisms are identical in both organ spaces and bile.

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Core tip: This study aimed to evaluate the effect of preoperative biliary drainage and bile contamination on the outcomes of patients who undergo pancreaticoduodenectomy (PD). The database of 4101 patients who underwent PD was reviewed. Preoperative biliary drainage was performed in 1964 patients (47.9%), and bile contamination was confirmed in 606 patients (14.8%). In patients undergoing PD, bile contamination is related to postoperative infectious complication including postoperative pancreatic fistula Grade B/C. The management of biliary contamination should be standardised for patients who require preoperative biliary drainage for PD, as the main microorganisms are identical in both organ spaces and bile.

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INTRODUCTION

Pancreatoduodenectomy (PD) is a common and complex procedure in gastroenterological surgery. Although the perioperative mortality rate of PD in high-volume centres is reportedly 1% to 2%, the post-PD morbidity rate remains relatively high at 20% to 50%[1-7]. In a previous study, we reported that infectious complications are the main cause of postoperative morbidity after PD[8]. Nine risk factors for infectious complications after PD were identified: Male sex, age of 70 years or more, body mass index of at least 25 kg/m^2, other previous malignancy, liver disease, bile contamination, surgery duration of 7 h or longer, intraoperative blood transfusion, and soft pancreas. Among these factors, bile contamination is the one that surgeons could control by appropriate perioperative management.

Obstructive jaundice is the most common symptom in patients with periampullary malignancy. Routine preoperative biliary drainage in patients undergoing surgery for cancer of the pancreatic head increases the rate of complications[9,10]. With the advent of neoadjuvant chemotherapy used to downstage potentially unresectable tumours in the hope of improving the outcome[11,12], concern regarding preoperative biliary drainage during neoadjuvant treatment is clinically relevant. Preoperative endoscopic biliary procedures are widespread in the management of periampullary tumours[13]. The effect of endoscopic procedures on biliary contamination and the immediate outcomes of PD remain controversial, although the several studies reported increased mortality or morbidity rate[14-17]. This study aimed to identify the clinical features and outcomes after PD in patients with infected bile based on data from the Japanese Society of Pancreatic Surgery for future management of perioperative infectious complications.

MATERIALS AND METHODS

A nationwide multi-institutional analysis of infectious complications after major pancreatic surgery was conducted by the Japanese Society of Pancreatic Surgery. A database of 4101 patients who underwent PD during a 3-year period were analysed for this study. This study was approved by the Institutional Ethics Committee of Kagawa University.

Definitions

The definitions of complications including infectious complications are almost identical to those of the American College of Surgeons–National Surgical Quality Improvement Program criteria (NSQIP)[18]. In the present study, infectious complications are defined as postoperative global infectious complications including

surgical site infection [*i.e.*, wound infection, intra-abdominal abscess, infected postoperative pancreatic fistula (POPF)] and extraparieto-abdominal infection (*i.e.*, catheter infection, pneumonia, urinary tract infection). Infectious complications are also identified as a specific clinical condition that was related to infection by bacteria, fungus, or virus in a specific organ/compartment. A positive culture without correlation to a specific clinical condition was not considered an infectious complication.

As the NSQIP 30-d mortality rates underestimate the mortality rate for complicated surgical procedures such as PD[7], the present study applied in-hospital mortality. In-hospital mortality was defined as death before postoperative day 30, and death among patients who were hospitalised for 30 d or more after surgery and died during that time[7].

Complication severity was graded according to the Clavien–Dindo classification[19]. Pancreatic fistula was defined according to the International Study Group on Pancreatic Fistula guidelines[20] as an amylase level in the drainage fluid on postoperative day 3 that is > 3 times the normal serum amylase level. Grade A fistulas presented with elevated drain amylase levels only, and they lacked any clinical consequences. Grade B fistulas, requiring therapeutic interventions, behaved in an intermediate fashion, with marginal increases in duration of hospitalisation and rates of complications. Grade C fistulas were the most severe, and patients frequently required intensive care unit transfer for sepsis management. An infected pancreatic fistula was defined as a clinically relevant fistula with proven infection by positive culture. Postoperative intra-abdominal haemorrhage was defined as bleeding requiring a blood transfusion, reoperation, or interventional radiology. An intra-abdominal abscess was defined as intra-abdominal fluid collection with positive cultures or organ/space surgical site infection in the abdominal cavity. A positive culture was not required to determine the presence of an infection, in cases in which NSQIP criteria were met and the clinical picture was consistent. Cultured organisms from organ space infections were determined by positive culture from the percutaneous drain, in patients with a clinical picture consistent with infection.

The types of biliary drainage and the results of preoperative bile culture were recorded for patients who underwent preoperative biliary drainage before PD. The preoperative biliary culture was performed in 1651 of 1964 patients (84.1%) who underwent biliary drainage in present study. Percutaneous trans-hepatic biliary drainage and endoscopic naso-biliary drainage were categorised as types of external drainage, and endoscopic retrograde biliary drainage was categorised as internal drainage. Positive results of cultured microorganisms in bile from a preoperative biliary stent or intraoperative bile collection were defined as bile contamination. Results of cultured microorganisms from overall infection site or organ space infections were collected from the patients with infectious complications. The standard perioperative management strategies were described previously[8]. Drains were usually removed at 3 to 7 postoperative days according to the early removal policy.

Statistical analysis

All statistical analyses were performed using SAS 8.2 (SAS Institute Inc., Cary, NC, United States). Patient characteristics and clinical factors were compared using the Mann-Whitney *U* test for continuous variables and Fisher's exact test or the chi-squared test for categorical variables. Risk factors that were significantly associated with POPF Grade B/C in univariate models ($P < 0.05$) were included in a multivariate logistic regression model. Throughout this study, P values < 0.05 were considered statistically significant.

RESULTS

The median age of the 4101 patients included in this study was 68 years (range 6-89); 1920 patients (46.8%) were over 70 years old. The male to female ratio was 1.53:1. Preoperative biliary drainage was performed in 1964 of 4101 patients (47.9%), and bile contamination was confirmed in 606 patients (14.8%).

Primary disease and infectious complications

The primary disease was significantly associated with preoperative biliary drainage and bile contamination (Table 1). Patients with extrahepatic bile duct carcinoma, ampulla of Vater carcinoma, and pancreatic carcinoma had a high frequency of preoperative biliary drainage (82.9%, 54.6%, and 5.80%) and bile contamination (34.3%, 26.2%, and 20.2%). In contrast, patients with intraductal papillary mucinous neoplasm, pancreas neuroendocrine tumour, and pancreas cystic tumour had a low

frequency of preoperative biliary drainage and bile contamination at 10% or less.

Background, outcomes, infectious complications, and cultured organisms

There were significant differences in the age and sex ratio in patients with or without preoperative biliary drainage and bile contamination (Table 2). The incidence of postoperative infectious complications was 37.9% in patients with preoperative biliary drainage and 42.4% in patients with biliary contamination, respectively. Preoperative biliary drainage was performed in male and elderly patients frequently. Bile contamination was also confirmed in male and elderly patients frequently. Preoperative biliary drainage and bile contamination were not associated with the rate of readmission and mortality. Bile contamination was associated with prolonged surgery duration. Preoperative biliary drainage and bile contamination were associated with both overall complications and infectious complications. Preoperative biliary drainage was associated with wound infection. Bile contamination was associated with POPF Grade B/C, wound infection, and catheter infection.

Cultured organisms from the bile and organ space

The most commonly cultured organisms from the bile were *Enterococcus* (42.7%), *Klebsiella* (26.6%), *Enterobacter* (14.2%), *Staphylococcus* (12.7%), and *E. Coli* (11.9%) (Table 3). The most commonly cultured organisms from the organ space (n = 596) were *Enterococcus* (47.7%), *Enterobacter* (20.0%), *Klebsiella* (14.8%), *Pseudomonas* (13.8%), and *Staphylococcus aureus* (methicillin-resistant *S. aureus*) (10.6%). These organisms were mainly cultured from drain discharge (n = 398) and intra-abdominal abscesses (n = 201 patients) which were strongly suspected to be associated with pancreatic fistula. The three most commonly cultured microorganisms from bile (*Enterococcus, Klebsiella, and Enterobacter)* were identical to those isolated from organ spaces. Most of the participating institutions (49 of 69 institutions) changed their antibiotic prophylaxis based on bile culture results in the present study.

Risk factors influencing POPF Grade B/C

Table 4 shows the results of multivariate analysis using risk factors that were significantly associated with POPF Grade B/C in univariate models. Six significant risk factors for infectious complications after PD were identified by multivariate analysis: male sex, age ≥ 70 years, body mass index ≥ 25 kg/m^2, bile contamination, soft pancreas, and operative time ≥ 7 h. Preoperative biliary drainage was not independent significant risk factor.

The 1283 patients (40.5%) with high total bilirubin level (< 1.0g/dL) were compared with the 1886 patients (59.5%) with normal total bilirubin level (> 1.0g/dL) for incidence of all POPF and clinical relevant POPF (Grade B/C). There was no significant difference for all POPF (37.8% *vs* 39.5%, P = 0.55) or clinical relevant POPF (21.4% *vs* 20.6%, P = 0.82) between the patients with high and normal total bilirubin levels.

Outcome according to the type of drainage

Table 5 shows the demographic characteristics, perioperative variables, and immediate outcome according to the type of drainage (external or internal drainage) in 1942 patients who received PD. External drainage was performed in 772 patients (endoscopic nasobiliary drainage in 499 cases and percutaneous transhepatic biliary drainage in 273 cases) and internal drainage (endoscopic retrograde biliary drainage) was performed in 1170 patients. The duration of surgery was significantly longer in the patients with internal drainage than in those with external drainage. There were no significant differences between the two groups concerning the incidence of postoperative complications such as infectious complication, POPF, delayed gastric emptying, and intra-abdominal bleeding.

DISCUSSION

In this multicentre observational study, preoperative biliary drainage and bile contamination had a notable effect on the immediate outcomes after PD, with a high frequency of infectious complications. Especially, bile contamination had a strong association with POPF (Grade B/C). Bile contamination was present mainly in patients with pancreas cancer, bile duct carcinoma, and ampulla of Vater carcinoma. Furthermore, we found that the three most commonly cultured microorganisms from bile (*Enterococcus, Klebsiella, and Enterobacter)* were identical to those isolated from organ spaces. As the post-PD morbidity rate remains considerably high[1-7], the prevention of bile contamination should be the most effective target to decrease the high morbidity after PD.

Table 1 Primary disease and bile contamination in patients who received pancreaticoduodenectomy, *n* (%)

	Preoperative biliary drainage		*P* value	Bile contamination		*P* value
	Yes (*n* = 1964)	No (*n* = 2137)		Yes (*n* = 606)	No (*n* = 2130)	
Disease			< 0.0001			< 0.0001
Pancreatic cancer	955 (50.8)	925 (49.2)		261 (20.2)	1029 (79.8)	
Bile duct carcinoma	691 (82.9)	143 (17.1)		208 (34.3)	399 (65.7)	
Intraductal papillary mucinous neoplasm	19 (4.5)	406 (95.5)		10 (5.1)	187 (94.9)	
Ampulla of Vater carcinoma	250 (54.6)	208 (45.4)		78 (26.2)	220 (73.8)	
Pancreas neuroendocrine tumour	11 (8.9)	113 (91.1)		3 (4.2)	69 (95.8)	
Pancreas cystic tumour	3 (2.6)	113 (97.4)		1 (2.9)	34 (97.1)	
Duodenal cancer	23 (18.3)	103 (81.7)		7 (9.7)	65 (90.3)	

Several studies showed that early surgery without preoperative biliary drainage is the standard treatment in patients with resectable pancreatic head cancer presenting with jaundice[9,10]. However, early surgery is not always feasible, and preoperative biliary drainage may be still necessary for patients with high hyper-bilirubinaemia at diagnosis or for those undergoing neoadjuvant treatment. It is still controversial how biliary drainage-related complications affect the incidence of postoperative complications after PD. Jagannath *et al*[21] reported that a positive intraoperative bile culture was associated with higher morbidity rates after PD, and biliary drainage was not associated with increased morbidity. Cortes *et al*[22] also reported that bile contamination had a remarkable effect on the immediate outcomes after PD for tumours, with a higher rate of infectious complications including wound and intraabdominal abscesses. Kitahata *et al*[23] reported that patients undergoing internal drainage had a significantly higher incidence of cholangitis because of biliary drainage (22.4% *vs* 1.7% in the external drainage group). Internal drainage significantly increased the incidence of morbidity compared with external drainage (41.8% *vs* 22.3%). The present study analysed 772 and 1170 patients who received external and internal drainages, respectively, and no significant difference in postoperative complications was found between the internal and external drainage groups. The results suggested that the postoperative infectious complications for patients who underwent PD were not associated with type of biliary drainage.

The incidence of positive bile culture was reported to increase significantly in patients who underwent biliary drainage and presented complications such as cholangitis[22]. Yanagimoto *et al*[24] reported that preoperative cholangitis after biliary drainage was associated with development of POPF Grade B/C. The present study clearly revealed that significant association of bile contamination and POPF Grade B/C. The results strongly supported previous reports[22,24]. Stent occlusion was reported to cause preoperative cholangitis, and cholangitis occurred in 26% of patients who underwent internal drainage[9]. A possible mechanism to explain the association between cholangitis and internal drainage is the ascent of microorganisms from the open passage to the duodenum and subsequent reflux of duodenal contents[25,26]. However, internal biliary drainage permits physiological bile flow, which is important for intestinal immunity and the prevention of bacterial translocation[27-29]. Several studies reported that metalic stents have more advantages compared with plastic stents when used for preoperative biliary drainage in patients undergoing neoadjuvant therapy for pancreatic cancer[30-32]. In two previous studies, stent-related complications were significantly higher with plastic stents than with fully covered self-expandable metal stents with no differences in the rate of overall surgical complications[33,34]. Further studies are required to assess the fully covered self-expandable metal stents as preoperative biliary drainage affects the surgical procedure or perioperative outcome.

To our knowledge, this is the first report that clarified the specific causative microorganism profile for bile contamination in a large PD series. The *Enterococcus*, *Enterobacter*, and *Klebsiella* species were the more commonly cultured microorganisms from organ space infections and bile contamination. The illustration of different organisms is useful for selecting prophylactic antibiotics or considering drain management after pancreatic surgery. In addition, there were significant differences in the incidence of bile contamination among primary diseases. The results of cultured organisms suggest the need for tailored antibiotic prophylaxis for patients with a high risk of biliary contamination. In the present study, preoperative biliary culture was

Table 2 Association of preoperative biliary drainage and bile contamination with immediate outcome after pancreaticoduodenectomy, *n* (%)

	Preoperative biliary drainage		*P* value	Bile contamination		*P* value
	Yes (*n* = 1964)	No (*n* = 2137)		Yes (*n* = 606)	No (*n* = 2130)	
Demographics						
Age (yr), median	69	68	< 0.0001	69	68	0.0004
Sex ratio (M:F)	1.81:1	1.34:1	< 0.0001	2.11:1	1.56:1	0.0012
Duration of hospital stay (d), median	29	29	0.29	29	31	0.11
Readmission	64 (3.3)	84 (3.9)	0.25	19 (3.1)	91 (4.3)	0.33
In-hospital death	42 (2.1)	34 (1.6)	0.21	8 (1.3)	46 (2.2)	0.19
Operative variables						
Estimated blood loss (g), median	855	643	< 0.0001	875	759	0.053
Duration of surgery (min), median	487	461	< 0.0001	497	483	0.0005
Postoperative complications						
Overall complications	1084 (55.2)	1114 (52.1)	0.049	356 (58.7)	1130 (53.1)	0.0014
Infectious complications	744 (37.9)	714 (33.4)	0.003	257 (42.4)	746 (35.0)	0.0003
Severe complications (grade III or more)	340 (17.3)	316 (14.8)	0.036	110 (18.2)	321 (15.0)	0.039
POPF (all)	739 (37.6)	809 (37.9)	0.42	246 (40.6)	773 (36.3)	0.06
Delayed gastric emptying	111 (5.7)	144 (6.7)	0.18	40 (6.6)	143 (6.7)	0.42
Intra-abdominal bleeding	67 (3.4)	57 (2.7)	0.16	18 (3.0)	61 (2.9)	0.78
Details of infectious complication						
POPF (ISGPF grade B or C)	444 (22.6)	438 (20.5)	0.13	154 (25.4)	432 (20.3)	0.003
Wound infection	320 (16.3)	216 (10.3)	< 0.0001	93 (15.3)	263 (12.3)	0.045
Intra-abdominal abscess	289 (14.7)	295 (14.0)	0.53	94 (15.5)	293 (13.8)	0.23
Cholangitis	79 (4.1)	95 (4.5)	0.45	24 (4.0)	105 (4.9)	0.35
Pneumonia	61 (3.1)	61 (2.9)	0.7	21 (3.5)	66 (3.1)	0.62
Liver abscess	21 (1.1)	24 (1.2)	0.83	9 (1.5)	19 (0.9)	0.21
Sepsis	86 (4.5)	86 (4.2)	0.66	30 (5.0)	83 (3.9)	0.24
Pseudomembranous enteritis	31 (1.6)	30 (1.4)	0.68	13 (2.1)	27 (1.3)	0.12
Catheter infection	91 (4.7)	115 (5.5)	0.24	41 (6.8)	98 (4.6)	0.029
Fungaemia	28 (1.5)	28 (1.4)	0.8	8 (1.3)	25 (1.2)	0.75

The variables were identical to those of the American College of Surgeons–National Surgical Quality Improvement Program. POPF: Postoperative pancreatic fistula; ISGPF: Influencing postoperative pancreatic fistula.

performed in 1651 of 1964 patients (84.1%) who underwent biliary drainage. Bile contamination was confirmed in 606 of 1651 patients (36.7%). Most of the participating institutions (49 of 69 institutions) changed their antibiotic prophylaxis based on bile culture results in the present study. As the specific antibiotic prophylaxis based on bile culture results prevents infectious complications in PD patients with preoperative biliary drainage[35], preoperative bile culture should be considered in patients with biliary drainage. However, as there is currently no consensus regarding the appropriate type of antibiotic prophylaxis, a prospective study is warranted to provide evidence to validate appropriate antibiotic prophylaxis for patients with biliary contamination.

This multicentre study has several limitations. First, data were retrospectively collected, which makes it a potential source for significant bias. Second, the results may have been influenced by hospital volume, hospital training status, hospital compliance, and procedure-specific variables. Third, in some patients who received immediate internal drainage, a preoperative biliary culture was not obtained. Although these limitations are recognised, we believe that our findings will contribute to improving quality control in pancreatic surgery. Further prospective, randomised studies are needed to overcome these limitations.

In conclusion, preoperative biliary drainage and bile contamination had a notable effect on immediate outcomes after PD, with high frequency of infectious complications. Particularly, bile contamination is related to POPF Grade B/C. Management of biliary contamination should be standardised for patients who

Table 3 Comparison of cultured organisms from bile and organ space infections

Characteristic	*n* (%)
Cultured from bile	606
Enterococcus	259 (42.7)
Klebsiella	161 (26.6)
Enterobacter	86 (14.2)
Streptococcus	77 (12.7)
E.coli	72 (11.9)
Other Gram negative rods	59 (9.7)
Citrobacter	42 (6.9)
Pseudomona	38 (6.3)
Coagulase negative staphyloccoccus	34 (5.6)
Candida albicans	23 (3.8)
Staphylococcus aureus (MRSA)	20 (3.3)
Staphylococcus aureus (MSSA)	10 (1.7)
Cultured from organ space	596
Enterococcus	284 (47.7)
Enterobacter	119 (20.0)
Klebsiella	88 (14.8)
Pseudomona	82 (13.8)
Staphylococcus aureus (MRSA)	63 (10.6)
Candida albicans	58 (9.7)
Coagulase-negative Staphylococcus	55 (9.2)
Streptococcus	51 (8.6)
Staphylococcus aureus (MSSA)	48 (8.1)
E.coli	26 (4.4)

MRSA: Methicillin-resistant *S. aureus*; MSSA: Methicillin-sensitive *S. aureus*.

require preoperative biliary drainage for PD, as the main microorganisms are identical in both infected POPF and bile. These findings contribute to the proper management of patients with biliary drainage for PD and may help to establish perioperative therapeutic strategies for biliary contaminations.

Table 4 Multivariate analysis for risk factors influencing postoperative pancreatic fistula (Grade B/C) patients who received pancreaticoduodenectomy

Risk factor	Significance (*P* value)	Odds ratio	95%CI
Male sex	< 0.0001	1.815	1.459-2.266
Age ≥ 70	0.032	1.250	1.018-1.535
BMI (kg/m^2) ≥ 25	< 0.0001	2.095	1.610-2.718
Other previous malignancies	0.079	1.253	0.971-1.612
Liver disease	0.119	1.422	0.903-2.200
Preoperative biliary drainage	0.461	1.087	0.869-1.361
Bile contamination	0.026	1.338	1.033-1.729
Soft pancreas	< 0.0001	4.594	3.650-5.824
Operation time (h) ≥ 7	0.0021	1.441	1.143-1.822

BMI: Body mass index; NA: Not available; POPF: Postoperative pancreatic fistula; ISGPF: International Study Group on Pancreatic Fistula; CI: Confidence interval.

Table 5 Comparison of complications and immediate outcome according to the type of drainage (external or internal) after pancreaticoduodenectomy, *n* (%)

	Type of biliary drainage		*P* value
	External (*n* = 772)	Internal (*n* = 1170)	
Demographics			
Age (yr), median	64	62	0.025
Sex ratio (M:F)	1.97:1	1.84:1	0.99
Duration of hospital stay (d), median	30	28	0.72
Readmission	29 (3.8)	36 (3.1)	0.32
In-hospital death	15 (1.9)	27 (2.3)	0.58
Operative variables			
Estimated blood loss (g), median	855	860	0.75
Duration of surgery (min), median	475	500	0.0004
Postoperative complications			
Overall complications	433 (56.1)	646 (55.2)	0.7
Infectious complications	293 (38.0)	445 (38.0)	0.77
Severe complications (grade III or more)	127 (16.5)	211 (18.0)	0.35
POPF (all)	284 (36.8)	450 (38.5)	0.57
POPF (ISGPF grade B or C)	164 (21.2)	277 (19.4)	0.19
Delayed gastric emptying	97 (12.6)	166 (14.2)	0.37
Intra-abdominal bleeding	36 (4.7)	68 (5.8)	0.32

The variables were identical to those of the American College of Surgeons–National Surgical Quality Improvement Program Percutaneous transhepatic biliary drainage and endoscopic nasobiliary drainage were categorized as the types of external drainage and endoscopic retrograde biliary drainage was categorized as internal drainage. External drainage was performed in 772 patients (endoscopic nasobiliary drainage in 499 cases, percutaneous transhepatic biliary drainage in 241 cases, and PTGBD in 32 cases) and internal drainage was performed in 1170 patients. POPF: Postoperative pancreatic fistula.

ARTICLE HIGHLIGHTS

Research background

Preoperative endoscopic biliary procedures are widespread in the management of periampullary tumours. The influence of bile contamination on the infectious complications of patients undergoing pancreaticoduodenectomy (PD) has not been thoroughly evaluated.

Research motivation

The large data of clinical features and outcomes after PD in patients with infected bile will help improve future clinical outcome.

Research objectives
This study aimed to identify the clinical features and outcomes after PD in patients with infected bile based on data from the Japanese Society of Pancreatic Surgery for future management of perioperative infectious complications.

Research methods
We retrospectively reviewed the database of 4101 patients who underwent PD. Preoperative biliary drainage was performed in 1964 patients (47.9%), and bile contamination was confirmed in 606 patients (14.8%).

Research results
The incidence of postoperative infectious complications was 37.9% in patients with preoperative biliary drainage and 42.4% in patients with biliary contamination, respectively. Patients with extrahepatic bile duct carcinoma, ampulla of Vater carcinoma, and pancreatic carcinoma had a high frequency of preoperative biliary drainage (82.9%, 54.6%, and 50.8%) and bile contamination (34.3%, 26.2%, and 20.2%). Bile contamination was associated with postoperative pancreatic fistula (POPF) Grade B/C, wound infection, and catheter infection. A multivariate logistic regression analysis revealed that biliary contamination (odds ratio 1.33, $P = 0.027$) was the independent risk factor for POPF Grade B/C. The three most commonly cultured microorganisms from bile (*Enterococcus, Klebsiella, and Enterobacter)* were identical to those isolated from organ spaces.

Research conclusions
In patients undergoing PD, bile contamination is related to postoperative infectious complication including POPF Grade B/C.

Research perspectives
The management of biliary contamination should be standardised for patients who require preoperative biliary drainage for PD, as the main microorganisms are identical in both organ spaces and bile.

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ORIGINAL ARTICLE

Observational Study

Direct costs of carcinoid syndrome diarrhea among adults in the United States

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Institutional review board statement: We conducted a retrospective cohort study using the IBM® MarketScan® Database, a commercial health insurance claims database for employer-insured beneficiaries in the United States. The database is fully compliant with the Health Insurance Portability and Accountability Act and meets the criteria for a limited-use dataset. Since the patient and provider data included in this analysis were fully de-identified, this study was exempt from the Institutional Review Board review.

Informed consent statement: This study involved analyses of a Health Insurance Portability and Accountability Act-compliant secondary database, the IBM® MarketScan® Database, thus no informed consent was feasible or required.

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Abstract

BACKGROUND

The burden of carcinoid syndrome (CS) among patients with neuroendocrine tumors is substantial and has been shown to result in increased healthcare resource use and costs. The incremental burden of CS diarrhea (CSD) is less well understood, particularly among working age adults who make up a large proportion of the population of patients with CS.

AIM

To estimate the direct medical costs of CSD to a self-insured employer in the United States.

METHODS

CS patients with and without CSD were identified in the IBM® MarketScan® Database, including the Medicare Supplemental Coordination of Benefits database. Eligible patients had ≥ 1 medical claim for CS with continuous health plan enrollment for ≥ 12 mo prior to their first CS diagnosis and for ≥ 30 d after, no claims for acromegaly, and no clinical trial participation during the study period (2014-2016). Baseline demographic and clinical characteristics, including comorbidities and treatment, were analyzed using descriptive statistics. Measures of healthcare resource use and costs were compared between patients with and without CSD, including Emergency Department (ED) visits, hospital admissions and length of stay, physician office visits, outpatient services, and prescription claims, using univariate and multivariate analyses to evaluate associations of CSD with healthcare resource use and costs, controlling for baseline characteristics.

RESULTS

Overall, 6855 patients with CS were identified of which 4,043 were eligible for the

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Data sharing statement: The statistician for this study, Samyukta Dharba, conducted all statistical analyses using a Health Insurance Portability and Accountability Act-compliant secondary database, the IBM® MarketScan® Database.

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analysis (1352 with CSD, 2691 with CS only). Baseline demographic and clinical characteristics were similar between groups with the exception of age, underlying tumor type, and health insurance plan. Patients with CSD were older, had more comorbidities, and received more somatostatin analog therapy at baseline. Patients with CSD required greater use of healthcare resources and incurred higher costs than their peers without CSD, including hospitalizations (44% *vs* 25%) and ED visits (55% *vs* 31%). The total adjusted annual healthcare costs per patient were 50% higher (+ $23865) among those with CSD, driven by outpatient services (+ 56%), prescriptions (+ 48%), ED visits (+ 26%), physician office visits (+ 21%), and hospital admissions (+ 11%).

CONCLUSION
The economic burden of CSD is greater than that of CS alone among insured working age adults in the United States, which may benefit from timely diagnosis and management.

Key words: Carcinoid syndrome; Carcinoid syndrome diarrhea; Healthcare costs; Neuroendocrine tumors

Core tip: Healthcare resource use and costs among patients with carcinoid syndrome (CS) are known to be high, but the incremental burden of CS diarrhea (CSD) is less well understood. We analyzed insured, working age CS patients with and without CSD using the MarketScan® database (2014-2016) and observed a greater economic burden in the presence of CSD. Patients with CSD required more healthcare resources than their peers without CSD, including hospitalizations (44% *vs* 25%) and Emergency Department visits (55% *vs* 31%). Total adjusted mean annual costs per patient were 50% higher (+ $25865), driven largely by the use of more outpatient services (+56%).

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INTRODUCTION

Neuroendocrine tumors (NETs), formerly known as carcinoid tumors, are potentially functional secretory tumors that arise in neuroendocrine cells throughout the body, most often in the gastrointestinal system but also in the pancreas, lungs, and other organs[1-3]. NETs have an estimated incidence of 70 cases per 1 million people, with an increasing prevalence over the past 20 years due to improvements in identification and characterization[3-5]. NETs produce hormonal factors that induce carcinoid syndrome (CS), characterized primarily by diarrhea, flushing, hypotension, tachycardia, and bronchoconstriction that may include cardiovascular and/or pulmonary complications[6-8]. One-third (35%) of patients with NETs may develop CS, 80% of whom are likely to have associated diarrhea (CSD)[9,10].

Carcinoid syndrome has been shown to affect tumor characteristics and advancement, causing substantial morbidity and reducing patient quality of life and survival[11-14]. Nearly all (97%) CS patients in a recent clinical trial reported bowel movement-related issues at baseline along with flushing (83%), abdominal pain (63%), and low energy (63%), among other symptoms[12]. In addition to substantial clinical morbidity, patients with CSD require greater healthcare resources than their peers without diarrhea[14]. Working age adults with CSD have significantly more hospitalizations, Emergency Department (ED) visits, and CS-related office visits within 1 year of diagnosis compared to peers without diarrhea[15]. Given the rare nature of CS and CSD, the prevalence and burden of CSD among patients with NETs has not been well characterized. This study aimed to further characterize the direct costs of CSD in a population of insured adults in the United States.

MATERIALS AND METHODS

We conducted a retrospective cohort study of patients with CS or CSD from January 12014 through December 312016. Patients with a diagnosis of CS (ICD-9259.2; ICD-10 E34.0) with or without CSD (ICD-9 564.5, 787.91; ICD-10 K59.1, R19.7) were identified in the IBM® MarketScan® Database, including the Medicare Supplemental Coordination of Benefits database. Eligible patients were adults ≥ 18 years of age at the time of first CS medical claim (index) with continuous health plan enrollment 12 mo prior to the index date and ≥ 30 d after; no medical claim for acromegaly; and no clinical trial participation during the study period.

Baseline demographic and clinical characteristics were identified, including health insurance plan type, Charlson Comorbidity Index, comorbidities, and baseline CS or CSD treatment. Overall and CSD-related measures of healthcare resource use and costs included ED visits, hospital admissions and length of stay, physician office visits, outpatient services, and prescription claims.

Statistical analysis

Descriptive analyses were conducted using measures of central tendency including baseline demographic and clinical characteristics and treatment, and healthcare resource use and costs among patients with and without non-infectious diarrhea (CSD). Univariate analyses of baseline characteristics and outcomes between patients with and without CSD were performed. Student's *t*-test was used to analyze continuous variables and Chi-square tests for categorical variables. Multivariate analyses were performed using general linear models with appropriate distribution and link to evaluate associations of CSD with healthcare resource use and costs, controlling for baseline characteristics. All statistical tests were 2-sided unless stated otherwise with significance tests based on alpha ≤ 0.05, and 95% confidence intervals were calculated using 2-sided criteria. All statistical analyses were performed and reviewed by the biostatistician, Samyukta Dharba, and conducted using SAS version 9.4 (SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

Overall, 6855 patients with ≥ 1 medical claim for CS were identified during the study period, 4043 of whom (1352 with CSD, 2691 with CS only) were eligible for the analysis (Figure 1). Baseline demographic and clinical characteristics were similar between groups with the exception of age, underlying tumor type, and health insurance plan (Table 1). Patients with CSD were older, had more comorbidities, and received more somatostatin analog therapy (SSA) at baseline.

Healthcare resource use and costs

Patients with CSD required greater use of healthcare resources and incurred higher costs than their peers with CS only. More patients with CSD were hospitalized compared to those with CS only (44% *vs* 25%) and more patients with CSD had ED visits (55% *vs* 31%) during the study period (Table 2). The total adjusted annual healthcare costs per patient were 50% higher (+ $23865) among those with CSD, driven by outpatient services (+ 56%), prescriptions (+ 48%), ED visits (+ 26%), physician office visits (+ 21%), and hospital admissions (+ 11%; Table 3).

DISCUSSION

This study showed greater healthcare resource use and costs among patients with CSD compared with their peers with CS only. The overall baseline burden of CS was high in both cohorts, and healthcare utilization was driven by both hospitalizations and outpatient services.

These findings are consistent with those of others who have reported the economic burden of CS and CSD. Broder and colleagues recently conducted a similar study in adults < 65 years of age that reported more hospitalizations, ED visits and outpatient visits among patients with CSD compared to those without[15]. Adjusted annual costs were also higher among those with CSD (CSD, $81610 *vs* CS only, $51719), but lower than those observed in this study (CSD, $105153 *vs* CS only, $54701). Burton and Lapuerta analyzed medical claims for US adults with CS and inadequate symptom control from somatostatin analog therapy[16]. The proportion of patients with CSD-related ED visits and hospitalizations nearly doubled (9% to 16%) following escalation

Table 1 Baseline demographic and clinical characteristics

	CSD (*n* = 1352)	CS only (*n* = 2691)	*P* value
Age (yr)			
Mean (SD)	60.3 (13.8)	57.3 (13.9)	< 0.001
Sex, *n* (%)			
Female	799 (59)	1477 (55)	0.011
Male	553 (41)	1214 (45)	
Primary tumor site, *n* (%)			
Malignant carcinoid tumor of unspecified site	295 (22)	331 (12)	< 0.0001
Malignant carcinoid tumors of the small intestine	303 (22)	328 (12)	< 0.0001
Malignant carcinoid tumors of the appendix, large intestine, rectum	110 (8)	137 (5)	< 0.0001
Malignant carcinoid tumors of other sites	429 (32)	523 (19)	< 0.0001
Malignant poorly differentiated neuroendocrine tumors	168 (12)	216 (8)	< 0.0001
Other malignant neuroendocrine tumors	69 (5)	60 (2)	< 0.0001
Malignant neoplasm of endocrine pancreas	28 (2)	31 (1)	0.022
Region, *n* (%)			
South	535 (40)	1122 (42)	0.059
North Central	331 (24)	574 (21)	
Northeast	268 (20)	600 (22)	
West	205 (15)	367 (14)	
Unknown	13 (1)	28 (1)	
Metropolitan statistical area, *n* (%)			
Urban	1145 (85)	2364 (88)	0.005
Rural	206 (15)	327 (12)	
Employment status			
Active, full-time	577 (43)	1303 (48)	–
Active, part-time or seasonal	3 (0.2)	20 (1)	
Early retiree	465 (34)	717 (27)	
Other/unknown	307 (23)	651 (24)	
Health insurance plan type, *n* (%)			
PPO	794 (59)	1654 (61)	0.001
Comprehensive	200 (15)	286 (11)	
CDHP/HDHP	143 (11)	305 (11)	
HMO	123 (9)	195 (7)	
POS/POS with capitation	71 (5)	176 (7)	
EPO	7 (1)	36 (1)	
Missing/unknown	14 (1)	39 (1)	
Charlson comorbidity index, mean (SD)comorbidities	1.6 (3.5)	1.0 (2.9)	< 0.0001
Nausea/vomiting	411 (30)	294 (11)	< 0.0001
Flushing	70 (5)	46 (2)	< 0.0001
Asthma	147 (11)	228 (8)	0.013
Dyspnea/wheezing	364 (27)	546 (20)	< 0.0001
Cardiac palpitations	105 (8)	160 (6)	0.027
Hypotension	47 (3)	42 (2)	< 0.0001
Asthenia/fatigue	424 (31)	614 (23)	< 0.0001
Dizziness	184 (14)	248 (9)	< 0.0001
Intestinal complication	96 (7)	94 (3)	< 0.0001
Carcinoid heart disease	231 (17)	327 (12)	< 0.0001
Vascular condition	581 (43)	899 (33)	< 0.0001
Metastasis/secondary neoplasm	(9)	428 (16)	< 0.0001
Baseline treatment, *n* (%)			
Immediate release somatostatin analog	36 (3)	25 (1)	< 0.0001
Long-acting somatostatin analog, octreotide	427 (32)	429 (16)	< 0.0001
Long-acting somatostatin analog, lanreotide	18 (1)	17 (1)	0.024

Chemotherapy	84 (6)	104 (4)	0.001
Peptide receptor radionuclide therapy	232 (17)	277 (10)	< 0.0001
Ablative liver therapy	3 (0.2)	20 (1)	0.038
Targeted therapy	45 (3)	46 (2)	0.001

SD: Standard deviation; IQR: Interquartile range; PPO: Preferred provider organization; CDHP: Consumer-directed health plan; HDHP: High-deductible health plan; POS: Point-of-service; EPO: Exclusive provider organization; CS: Carcinoid syndrome; CSD: CS diarrhea.

of somatostatin analog therapy doses, considered a proxy for CS symptom severity, which incurred higher all-cause healthcare costs ($8305 *vs* $4116 per patient per month). Shen and colleagues reported higher total monthly costs among Medicare beneficiaries who developed CS within the first year of NET diagnosis compared with peers who did not develop CS ($4658 *vs* $3170)[14]. This average monthly cost was similar to our estimate in CS only patients ($4310), but the authors did not investigate the additional burden of CSD nor was the focus on the working age population. Our study has offered further insights into the burden of CS and CSD in a population of commercially insured working age adults in the United States.

This is the first study to our knowledge that evaluates the burden of CSD-related healthcare resource use and costs among commercially insured, working age adults with a focus on the employer and insurer perspective. In particular, this population may have fewer comorbid causes of morbidity and mortality than those observed in older populations such as Medicare beneficiaries. The database was limited to insured patients with available employment information which does not capture the burden of CS and CSD among uninsured patients or those without some employment-related information available. The retrospective analysis of data collected for insurance claims administration may also be vulnerable to classification issues related to the coding of patient characteristics and medical encounters, which we would not be able to see or account for within this database. For example, the coding of certain neuroendocrine tumor types such as "poorly differentiated" was observed in 11%-23% of patients and yet would be considered an unlikely source of CS based on prior data[11]. The prevalence and incremental costs of CSD among patients with CS may be underestimated since CSD is likely to be captured less often for billing purposes in an administrative claims database than for clinical assessment purposes in medical records.

Patients with NETs and CS suffer substantial burden and require notable healthcare resources with associated costs, particularly in the presence of CSD. This condition negatively impacts patients, employers, and the healthcare system. Timely identification and management of CSD in patients with CS may reduce the burden of this debilitating and resource-intensive condition.

Table 2 Healthcare resource use adjusted for baseline demographic and clinical characteristics

	CSD (*n* = 1352), mean (SD)	CS only (*n* = 2691), mean (SD)	*P* value[1]
Hospital admissions	0.8 (1.8)	0.4 (1.2)	< 0.0001
Length of hospital stay	5.3 (17.0)	2.3 (11.7)	< 0.0001
Physician office visits	16.3 (9.8)	12.0 (9.2)	< 0.0001
Emergency room visits	1.3 (3.1)	0.6 (1.6)	< 0.0001
Outpatient services	34.2 (28.4)	23.1 (23.2)	< 0.0001
Prescription claims	41.5 (33.1)	30.5 (27.6)	< 0.0001

[1]Significance of estimates from the generalized linear model with Poisson distribution and log link. Covariates included age (continuous), prior specific outcome, charlson comorbidity index, sex, health insurance plan type, region, and metropolitan statistical area. CS: Carcinoid syndrome; CSD: CS diarrhea.

Table 3 Healthcare costs

	CSD (*n* = 1352), mean (median)	CS only (*n* = 2691), mean (median)	*P* value[1]
Overall expenditures	$105153 ($63033)	$54701 ($16644)	< 0.0001
Hospitalization	$26361 ($0)	$13247 ($0)	0.11
Physician office	$2075 ($1653)	$1452 ($1060)	< 0.0001
Emergency room	$2666 ($192)	$994 ($0)	< 0.0001
Outpatient services	$59258 ($31218)	$32014 ($7735)	< 0.0001
Prescriptions	$14792 ($3401)	$6994 ($1361)	< 0.0001

[1]Significance of estimates from the generalized linear model with gamma distribution and log link. Covariates included age (continuous), prior specific cost for that category, charlson comorbidity index, sex, health insurance plan type, region, and metropolitan statistical area. CS: Carcinoid syndrome; CSD: CS diarrhea.

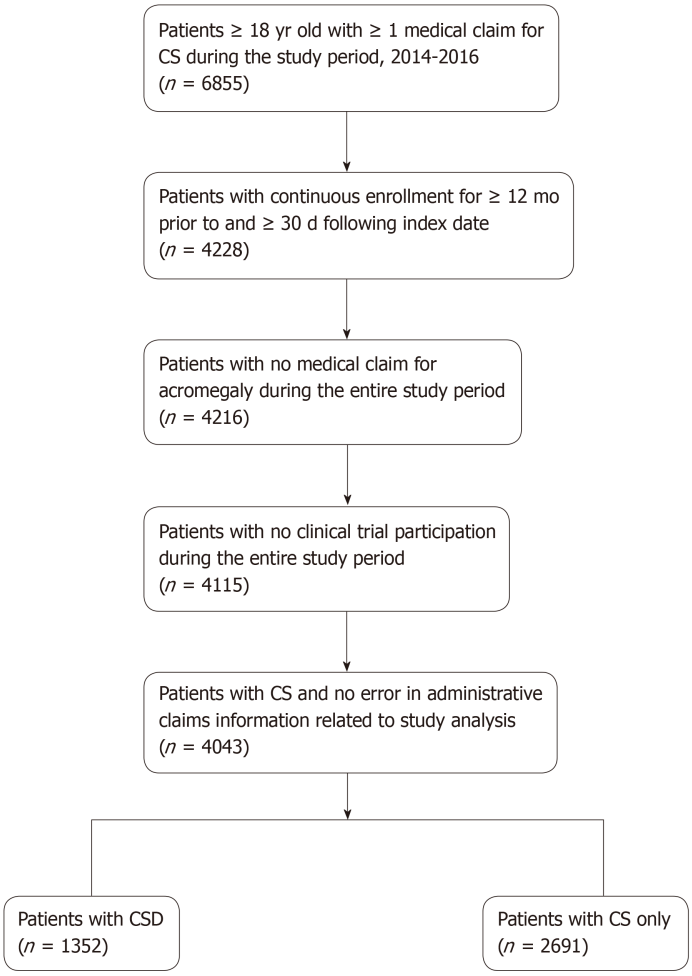

Figure 1 Patient attrition.

ARTICLE HIGHLIGHTS

Research background

Carcinoid syndrome (CS) in patients with neuroendocrine tumors has been shown to bear substantial economic costs to patients and healthcare systems; however, the incremental burden of CS diarrhea (CSD) has not been well characterized in the literature. Since patients with CSD are most often of working age, it is important to understand the direct costs of CSD from a population health management perspective. This study aims to provide detail and context related to the direct costs of CSD on insured working age adults in the United States.

Research motivation

Quantifying the economic burden of CSD in this population is important for setting healthcare priorities and allocating healthcare resources in an appropriate and efficient manner. Patients with CSD may be underserved due to a lack of information and insight regarding the burden of CSD overall, and payers need to understand the scope of economic burden of CSD in order to design effective policies and programs. Future research may validate these findings and apply similar methods to additional health system configurations such as integrated delivery networks, single payer systems, and other approaches to population health management found worldwide.

Research objectives

We aimed to quantify the incremental economic burden of CSD compared with patients who had CS but no CSD. The differentiation of CSD within the broader scope of CS costs is important for resource allocation and policy decisions, and has not been well studied. This objective allows population health managers to more clearly examine the additive costs that are specific to CSD in this patient population, where such discrimination of costs was not previously possible. Future research may build upon these insights and expand them to include indirect costs such as work productivity burden and other important factors.

Research methods

We conducted a retrospective study of CS patients with and without CSD as identified in the IBM® MarketScan® Database between 2014–2016, including the Medicare Supplemental Coordination of Benefits database. Patients had to have at least 1 medical claim for CS and continuous health plan enrollment for at least 12 mo prior to their first CS diagnosis, and for at least 30 d after. We excluded patients with documented claims for acromegaly, and those who participated in a clinical trial during the study period. Measures of healthcare resource use and costs were compared between patients with and without CSD, including Emergency Department (ED) visits, hospital admissions and length of stay, physician office visits, outpatient services, and prescription claims, using univariate and multivariate analyses to evaluate associations of CSD with healthcare resource use and costs, controlling for baseline characteristics. The methods applied in this analysis allowed us to distinguish the direct costs among patients with CS "only" from their peers had CS and CSD. This approach allowed us to characterize the additive, or incremental healthcare-related costs of CSD in the context of a patient population that was as similar as possible to those with CSD.

Research results

Our study identified 4043 patients with CS to be included in the analysis, 1352 with CSD and 2691 with CS only. Baseline demographic and clinical characteristics were similar between groups except that patients with CSD were older, had more comorbidities, and received more somatostatin analog therapy at baseline. Overall, patients with CSD required more healthcare resources and incurred higher costs than their peers with CS only. In particular, patients with CSD had more hospitalizations (44% *vs* 25%) and Emergency Department visits (55% *vs* 31%). When adjusted for baseline demographic and clinical characteristics, patients with CSD had higher mean healthcare resource use across all components of care, including hospital admissions (0.8 *vs* 0.4) and the mean length of stays (5.3 d *vs* 2.3 d), physician office visits (16.3 *vs* 12.0), Emergency Department visits (1.3 *vs* 0.6), outpatient services (34.2 *vs* 23.1), and prescription claims (41.5 *vs* 30.5). The total adjusted annual healthcare costs per patient were 50% higher (+ $23865) among those with CSD, driven by outpatient services (+ 56%), prescriptions (+ 48%), ED visits (+ 26%), physician office visits (+ 21%), and hospital admissions (+ 11%). These findings provide quantifiable differences in direct costs between patients with CS "only" and their peers who also have CSD. The increased costs observed across all avenues of care are indicative of the increased burden of CSD on patients and the healthcare resources needed to provide adequate care for this disruptive and damaging condition. Further research may validate these findings and investigate similar incremental costs in other healthcare settings.

Research conclusions

This study demonstrated that the costs of managing CSD are greater than those related to CS alone among insured working age adults in the United States, allowing population health managers to more intimately understand the incremental economic burden of CSD and to develop policies and programs accordingly. The methods applied in this study may be replicated or adopted to other data sources and healthcare settings to continue to characterize the additive costs of CSD in this predominantly working age population. This study provides a clear illustration of costs from the perspective of the employers and insurers, which is essential to effective policy and practice for this relatively young, active patient population. Timely identification and appropriate management of CSD may not only alleviate the clinical and humanistic burden of CSD to these patients, but may also reduce the economic burden of CSD to payers and population health managers.

Research perspectives

Patients with neuroendocrine tumors and CS require substantial healthcare resources to manage this condition, which are greatest among those who also have CSD. Supporting the timely identification and management of CSD should be a priority for population health managers, as this condition has been shown to negatively impact patients, employers, and the healthcare system. Future research may validate and extend these findings, and investigate indirect costs such as the impact of CS and CSD on quality of life, work productivity, and caregivers.

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SYSTEMATIC REVIEWS

Epidemiology of inflammatory bowel disease in South America: A systematic review

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Abstract

BACKGROUND

The worldwide epidemiology of inflammatory bowel disease (IBD) is rapidly changing. Increasing Crohn's disease (CD) and ulcerative colitis (UC) incidence and prevalence have been recorded in developing regions such as Asia, Africa and Eastern Europe where it was previously thought to be uncommon. Whether this is also the case in South America is not well known. Demonstration that developing regions worldwide have increasing IBD incidence would indicate that environmental change plays a significant role in the development of IBD.

AIM

To report the incidence, prevalence and disease characteristics of CD and UC within the South American continent.

METHODS

A systematic review was conducted by searching published studies in major international and regional databases (MEDLINE, EMBASE and Scopus) between January 1990 and December 2018. Outcomes considered were incidence, prevalence, phenotype, environmental and genetic factors, ethnicity and gender. A pair of independent reviewers screened and reviewed all identified articles.

RESULTS

One hundred and sixty two citations were initially retrieved with 18 studies included in this systematic review. The majority of included studies were from Brazil (n =13, 72%). The incidence of UC ranged from 4.3-5.3/100000 person-years whilst the incidence of CD ranged from 0.74-3.5/100000 person-years. Prevalence ranged from 15.0-24.1/100000 inhabitants for UC and from 2.4-14.1/100000 inhabitants for CD. The incidence and prevalence of both UC and CD has increased significantly in Brazil over the past 21 years. Pancolitis was the most common disease distribution in patients with UC whilst colonic involvement was the most common distribution in CD. People residing in urban

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areas were at higher risk of developing both CD and UC.

CONCLUSION
The IBD burden in South America is increasing at a rate possibly even greater than other developing regions around the world. There is a paucity of high-quality epidemiological studies and further robust and representative data are required to further explore modifiable risk factors and disease phenotypes.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; South America; Epidemiology

Core tip: The worldwide epidemiology of inflammatory bowel disease (IBD) is rapidly changing with increasing disease incidence and prevalence noted in developing regions such as Asia, Africa and Eastern Europe where it was previously thought to be uncommon. Whether this is the case in South America was previously not well known. Our systematic review demonstrates that the IBD burden in South America is precipitously increasing, particularly in industrialised regions. With a total population exceeding 400 million, the South American continent is expected to carry a significant proportion of the future global IBD burden.

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INTRODUCTION

Inflammatory bowel disease (IBD) refers to a chronic inflammatory disorder of the gastrointestinal tract which is thought to arise from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host[1]. The most common forms of IBD include ulcerative colitis (UC) and Crohn's disease (CD). UC characteristically involves the rectum and colon whereas CD may involve any part of the gastrointestinal tract however differentiation between these two conditions is not always clear[1,2]. Symptomatic disease is characterised by abdominal pain, diarrhoea, rectal bleeding, fever and weight loss which has a significant impact on quality of life[1,2].

IBD was first recognised in Western Europe during the industrial revolution and has encountered a rising incidence in this population since this time[3]. Whilst previously regarded exclusively as a disease of western nations, the epidemiology of IBD is rapidly changing worldwide. Although IBD incidence in developed areas such as Western Europe and the United States have been relatively stable, recent epidemiological studies suggest a significant increase in IBD incidence and prevalence in areas such as Asia, Africa and Eastern Europe where it was previously thought to be uncommon[3-9]. This epidemiological shift, seen in newly industrialised countries as well as in immigrant populations in western countries, is comparable to the patterns noted in western countries more than 50 years ago which occurred during a period of rapid socioeconomic development[4].

An increase in IBD incidence in developing nations has substantial implications for the understanding of IBD pathogenesis and environmental triggers. A recent Asia-Pacific Crohn's and Colitis epidemiology study demonstrated significant differences in risk factors and disease characteristics in IBD occurring in Asian *vs* Western populations[5]. Interestingly, in recent times the prevalence of CD appears to have caught up to the prevalence UC in the Asian population[5]. Furthermore, Asian patients tended to have a more severe CD phenotype at diagnosis[5]. Other notable findings included significant genetic heterogeneity between Asian and European patients as well as a protective effect of childhood antibiotic use in Asia for the development of IBD - a direct contrast to studies from Western nations[5].

Epidemiological data can provide valuable information about population-based

disease characteristics and burden which can also be utilised to anticipate healthcare needs. This is particularly important with chronic and incurable diseases such as IBD for which the concept of personalised medicine is paramount.

Recent efforts have been made to describe IBD in some developing nations within the South American continent. Further information about IBD in this region will help provide a valuable insight into the emerging epidemic of this disease within this population which will therefore enable the delivery of high-quality patient-centred care.

We therefore sought to report the incidence, prevalence and disease characteristics of CD and UC within the South American population.

MATERIALS AND METHODS

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines outlined in the PRISMA 2009 checklist[10].

Search strategy and selection criteria

Published studies in major international and regional databases (MEDLINE, EMBASE and Scopus) between January 1990 and December 2018 were searched. Search terms included Inflammatory Bowel Disease, Crohn, Crohn's disease, ulcerative colitis AND epidemiology, incidence, prevalence AND South America, Argentina, Chile, Uruguay, Brazil, Paraguay, Bolivia, Peru, Ecuador, Colombia, Venezuela, Suriname, Guayana, French Guayana.

Randomised controlled trials, cohort studies, cross-sectional studies, case control studies and observational studies were included if they included an adult population (> 18 years of age). Outcomes considered were incidence, prevalence, phenotype, environmental and genetic factors, ethnicity and gender. Included studies were restricted to those published in the English language only.

Screening and data extraction

A pair of independent reviewers screened titles and abstracts of all identified articles. Studies were initially sorted into one of the following categories: Excluded, low probability of inclusion, or high probability of inclusion. Studies deemed to be at low or high probability of inclusion were retrieved in their full-text version for further evaluation. Inclusion criteria was shown in Table 1.

RESULTS

This search strategy retrieved 160 citations, of which 35 were duplicates. Two further citations were identified by handsearching and a total of 73 were subsequently excluded by title and abstract. Out of the 54 full-text manuscripts retrieved for detailed evaluation, 18 met the inclusion criteria and were included in the systematic review as outlined in Figure 1.

Included studies were conducted in five of twelve countries within the South American continent - the majority from Brazil (n = 13, 72%). All included studies were hospital-based. The geographic area of South America covered by the included studies is outlined in Figure 2. The years of publication of included studies ranged from 1999 to 2018, with most studies being published within the last ten years (n = 15, 83%). Most included studies included patients with both UC and CD (n = 10, 56%). The main characteristics of the included studies are outlined in Table 2.

Incidence and prevalence

Six studies described the incdence of IBD in South America. Three were Brazilian studies that reported data from hospital records during different study periods: 1986 to 2005, 1988 to 2012 and 2012 to 2014[11-13]. The other three studies were conducted in Argentina, Columbia and Uruguay[14-16].

Victoria *et al*[11] conducted a retrospective registry-based study to investigate the incidence and prevalance of UC and CD in a specific region in the mid-western zone of Sao Paulo, Brazil. This study was conducted in four, five-year blocks over a 20 year time period (1986-2005) to compare changes over time. Both the incidence and prevalence of UC and CD was seen to increase during this time period (expressed as number of cases per 100000 inhabitants); UC Prevalence 0.99 to 15.0, CD Prevalence 0.24 to 5.7, annual UC Incidence 0.74 to 4.5, annual CD Incidence 0.24 to 3.5[11].

Increasing IBD incidence and prevalence within Brazil was also noted in a 21-year

Table 1 Inclusion criteria

Inclusion criteria	
Population	Adult (> 18 yr old)
Language	English
Date range	January 1990 to December 2018
Location	South America
Type of study	Randomised controlled trials, cohort studies, cross sectional studies, case series, and observational studies

retrospective study conducted by Parente *et al*[12] in the northeastern region of Brazil. This study, which included a total of 256 patients with IBD, demonstrated an increase in both combined IBD prevalence (1.2 to 21 per 100000 inhabitants) and combined annual IBD incidence (1.0 to 8.0 per 100000 inhabitants)[17]. Sub-analysis demonstrated that gradual increases occured in a similar pattern for both UC and CD during this time period[12].

As aforementioned, the incidence and prevalence of CD appears to be increasing over time, at least within the Brazilian population. A more recent retrospective study performed between 2012 and 2014 in the state of Espirto Santo, Brazil by Lima Martins *et al*[13] demonstrated a high incidence and prevalance of UC and CD (expressed as number of cases per 100000 inhabitants); annual UC Incidence 5.3, Prevalence 24.1 and annual CD Incidence 2.4, Prevalence 14.1. This higher than expected CD prevalence compared with other Brazilian studies, may potentially be explained by the high European immigrant population within this region[11,13]. Unfortunately, to date, there have been no studies to formally assess factors related to aetiopathogenesis within this specific region.

Similar prevalence data to that obtained in Brazil was also observed in a 2006 single-centred Columbian study by Barreto *et al*[14] which described UC and CD prevalences of 22 and 7 per 100000 inhabitants respectively within the city of Cartagena, Columbia. Interestingly, a prospective study by Buenavida *et al*[15] across five geographical areas of Uruguay between 2007 and 2008 also demonstrated a similar annual UC incidence to the Brazilian studies however CD appears to be less frequent with UC and CD incidences of 4.26 and 0.74 per 100000 inhabitants per year respectively.

IBD phenotype

All included studies demonstrated a significantly higher frequency of UC compared to CD within the South American population[11-28]. A single-centered prospective observational study conducted in Chile demonstrated a UC to CD ratio of 2.6:1 within 716 patients included in their study between 2012 and 2015[17]. In this study, pancolitis was the most common disease distribution in UC patients (n = 76, 50%) and colonic involvement was the most common distribution in CD (n = 44, 44%)[17]. In CD patients, the inflammatory subtype was most frequent (n = 80,80%) with perianal disease observed in 28% (n = 28)[17].

These phenotypic findings are also supported by the findings of Parente *et al*[12] in northeastern Brazil which demonstrated predominant CD features of; colonic disease location, nonstricturing and nonfistulizing disease behavior and a 25% frequency of perianal disease.

Environmental and genetic factors

The distribution of patients with IBD according to residence was evaluated by both Victoria *et al*[11] and Parente *et al*[12] with both studies demonstrating that the majority of patients with IBD lived in urban districts. In the study conducted by Victoria *et al*[11], 104 patients (90.5%) lived in an urban area compared to 11 (9.5%) who lived in a rural area, $P < 0.001$. Similar findings were demonstrated by Parente *et al*[12]; total IBD [86.1% (n = 217) *vs* 13.9% (n = 35), $P < 0.001$), CD [93.0% (n = 93) *vs* 7.0% (n = 7) $P < 0.001$] and UC [81.6% (n = 124) *vs* 18.4% (n = 28), $P < 0.001$].

A case-control study conducted by Salgado *et al*[18] at a statewide tertiary referral centre in Rio de Janeiro sought to identify envionmental risk factors associated with the development of CD to re-assess the hygeine hypothesis in this unique population. In this study, 145 outpatients with CD were compared to 163 controls by means of a 94-item survey regarding perinatal and childhood circumstances, living conditions, smoking and familial socioeconomic status. Controls were were recruited from caregivers of patients seen in different outpatient clinics at the same hospital. On univariate analysis, predictive variables for CD included male gender [Odds ratio

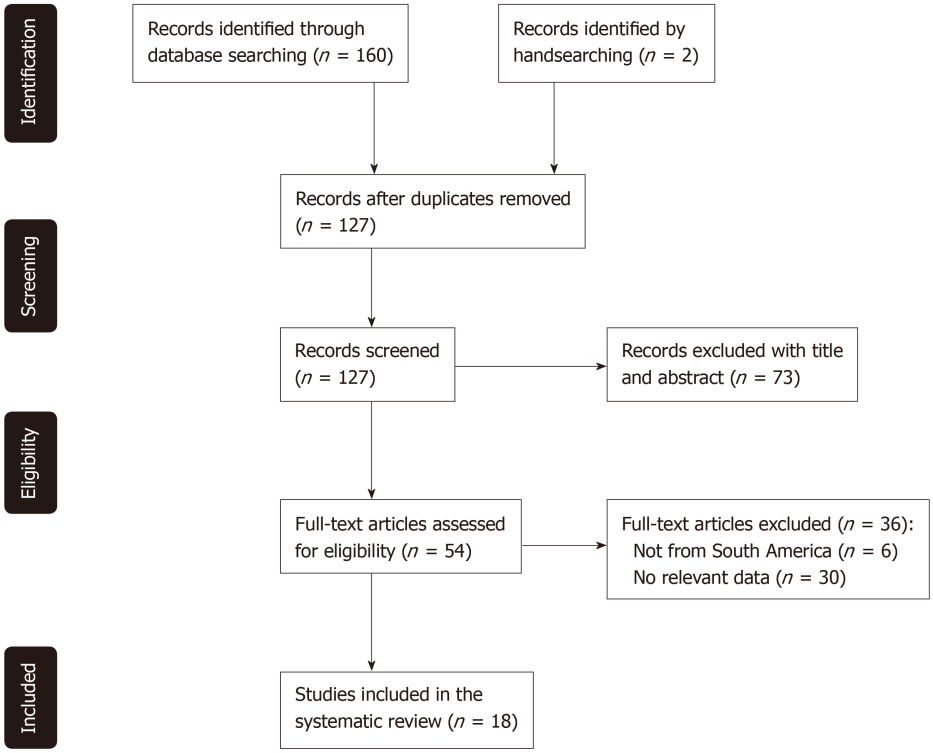

Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram.

(OR) = 2.09, P = 0.003], age under 40 (OR = 2.71, P < 0.001), "white" race (OR = 2.32, P = 0.002), small family in childhood (OR = 2.34, P < 0.006) and adulthood (OR = 3.02, P = 0.002), exposure to enteric pathogens (OR = 2.23, P = 0.001), and history of cigarette smoking (OR = 2.83, P = 0.002)[18].

The role of *Salmonella enterica* exposure in Chilean CD patients was examined by Alvarez-Lobos *et al*[19]. This single-centre case-control study compared 94 adult CD patients with 88 healthy age and sex matched controls[19]. Participants were analysed for exposure to *Salmonella enterica* and for their *NOD2/CARD15* gene status. Interestingly, no association between exposure to *Salmonella enterica* and CD was demonstrated in this study [16/94 (17%) *vs* 15/88 (17%), P = 0.8][19]. Seventeen CD patients (18%) had at least one mutation of the *NOD2/CARD15* gene however *NOD2/CARD15* gene status was not associated with *Salmonella enterica* exposure[19]. Queiroz *et al*[20] conducted the first (and only) South American study to exclusively examine associations among genetic polymorphisms with CD and UC. This landmark Brazilian study compared genetic polymorphisms in 43 patients with CD, 42 with UC and 541 controls. Data was analyzed in multivariate models adjusting for confounding factors. Queiroz *et al*[20] demonstrated positive associations between UC and proinflammatory polymorphisms at the *1L1RN* [OR = 2.43, 95% confidence interval (CI): 1.50-3.90, P < 0.001] and *TNFA-307* (OR = 1.70, 95%CI: 1.00-2.94, P < 0.001) loci as well as positive associations with CD and polymoprhisms in the *NOD2* gene (G908R; OR = 6.83, 95%CI: 1.62-25.45, P = 0.02 and L1007fsinsC, OR = 20.00, 95%CI: 3.21-124.69, P < 0.001).

Ethnicity

Ethnic background was also only explicitly evaluated within Brazil by the studies conducted by Victoria *et al*[11] and Parente *et al*[12]. Within the Sao Paulo region, Victoria *et al*[11] demonstrated a statistically significant predominence of combined IBD within Brazilians of European and Middle Eastern descent ("White" Brazilians) compared to Brazilians of African and Asiatic descent; 91.1% (n = 105) *vs* 8.0% (n = 9) *vs* 0.89% (n = 1), P < 0.001[2]. Parente *et al*[17] further classified ethncity into "Miscegenated", "White", "Black" and "Yellow" with the majority of CD, UC and combined IBD patients belonging to the miscegenated group; CD (64.0%, n = 64), UC (70.4%, 107), and combined IBD (67.9%, 171).

Gender

A non-statistically signficant female predominence for UC, CD and combined IBD

Table 2 Characteristics of included studies

Ref.	Country	Type of study	Type of IBD	Year data set	*n*	Average patient age	Specific group
Linares *et al*[16]	Argentina	Registry	UC/CD	1987-1993	39	38	Multicentre
Salgado *et al*[18]	Brazil	Case-control	CD	2017	145-163	NR	Single Centre
Lima Martins *et al*[13]	Brazil	Registry	UC/CD	2012-2014	1048	NR	Multicentre
Queiroz *et al*[20]	Brazil	Case-control	UC/CD	2017	85/541	40.0	Single Centre
Santos *et al*[21]	Brazil	Registry	UC/CD	2016	556	49.7	Single Centre
da Silva *et al*[22]	Brazil	Cross-sectional	UC	2011-2012	267	33.4	Multicentre
Parente *et al*[12]	Brazil	Registry	UC/CD	1988-2012	256	25.2	Multicentre
Victoria *et al*[11]	Brazil	Registry	UC/CD	1986-2005	115	38.0	Multicentre
Santana *et al*[23]	Brazil	Cross-sectional	CD	2006	65	37.3	Single Centre
Torres Udos *et al*[24]	Brazil	Cross-sectional	CD	1992-2007	90	33	Single Centre
Cohen *et al*[25]	Brazil	Cross-sectional	UC/CD	2008	50	42.2	Single Centre
Hardt *et al*[26]	Brazil	Cross-sectional	CD	2000-2012	175	35.5	Multicentre
Santana *et al*[27]	Brazil	Cross-sectional	CD	2005	47	38.5	Single Centre
de Barros *et al*[28]	Brazil	Cross-sectional	UC/CD	2012-2013	40	37.8	Single Centre
Simian *et al*[17]	Chile	Registry	UC/CD	2012-2015	716	36	Single Centre
Alvarez-Lobos *et al*[19]	Chile	Case-control	CD	2010-2012	94/90	35.5	Single Centre
Barreto *et al*[15]	Colombia	Registry	UC/CD	1991-2006	26	40	Single Centre
Buenavida *et al*[16]	Uruguay	Registry	UC/CD	2007-2008	34	40.7	Multicentre

UC: Ulcerative colitis; CD: Crohn's disease; IBD: Inflammatory bowel disease; NR: Not reported.

was demonstrated in the majority of included studies from Brazil and all included studies from Argentina, Uruguay, Chile and Columbia[11-15,17-28]. The gender distribution in UC and CD specifically, was best presented by Lima Martins *et al*[13] in Espirto Santo, Brazil. Of 669 patients with UC and 357 with CD, 60.80% (n = 407) and 54.60% (n = 195) were female (P = 0.16).

The aforementioned 20-year study conducted by Victoria *et al*[11] in Sao Paulo, Brazil was the only study to demonstrate a statistically significant higher incidence of total IBD among females (Male:Female RR = 0.44, P < 0.001). Interestingly, female predominence appeared to also be increasing with time over the four five-year study intervals between 1986 and 2005[11].

DISCUSSION

Our systematic review demonstrates that IBD is an expanding problem within the South American continent. The rising disease burden of IBD in South America appears to mirror the recent epidemiological shift observed in other developing nations such as Africa, Asia and Eastern Europe. While the incidence and prevalence of IBD in South America currently remains lower than western nations such as America, Australia and the United Kingdom, data obtained from the studied regions in Brazil, Argentina, Columbia and Uruguay demonstrate a significantly higher burden of disease compared to Asian countries such as Mainland China, Hong Kong, Indonesia, Malaysia, Singapore, Sri Lanka and Thailand[4-6,29]. With a combined population of over 430 million people, it is anticipated that South America will have carry a significant future burden of IBD worldwide[4].

Rampant industrialisation, including increased urbanisation, has resulted in a transformation of lifestyle behaviours and exposures which promote the development of IBD. Higher rates of cigarette smoking, sedentary occupations and lower breast feeding rates - all risk factors for the development of IBD, have been associated with adopted lifestyles in concentrated urban cities[29]. The overall IBD incidence and prevalence was consistently higher in the study conducted in Sao Paulo, Brazil by Victoria *et al*[11] compared to the data obtained from Piuai, Brazil by Parente *et al*[12]. These findings are not entirely surprising given the unique population demographics of each region; with Sao Paulo known to be a vibrant industrialised region with a robust IBD service while Piuai generally consists of a population with lower socioeconomic status and poor living conditions. Whilst underreporting in Piuai may potentially be contributory to this discrepancy, it is interesting to note that both of

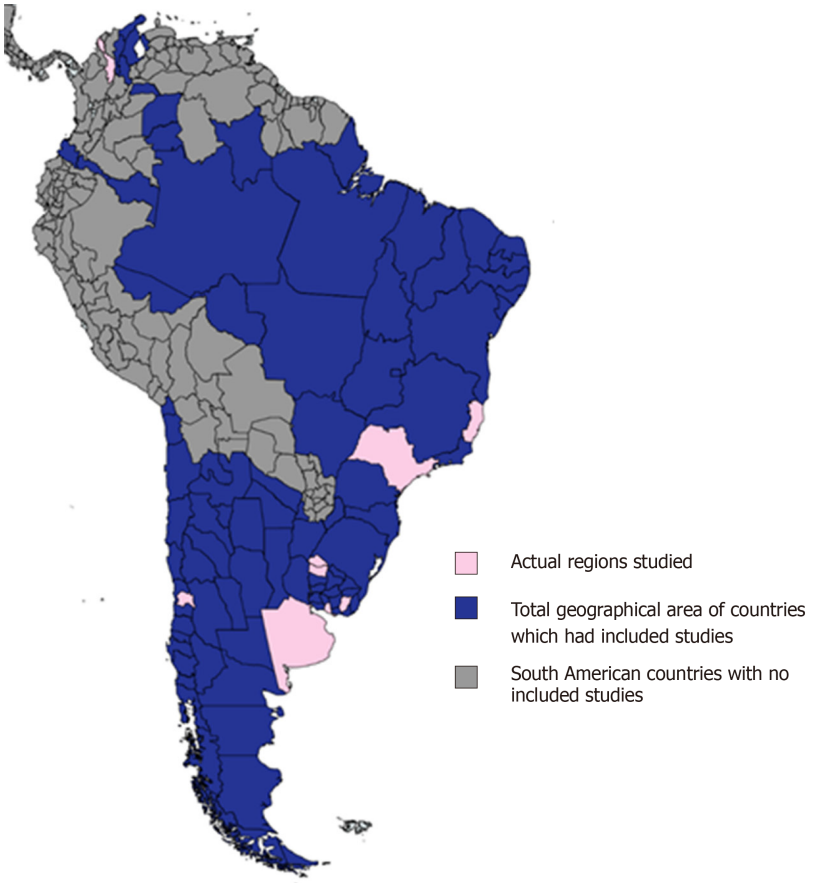

Figure 2 Geography of included studies within South America.

these studies have independently demonstrated that people are more likely to develop IBD if they lived within an urban district[11,12].

Based on the studies included in this systematic review, the population of IBD patients in South America appear to have a unique disease phenotype compared to other parts of the world. As observed in Asia, UC was more common than CD in all included studies from South America - a direct contrast to western prevalence data[5,7,8,11-28]. Previous reports from Asia, Australia, the United Kington and United States of America demonstrate a predominance of small bowel CD and rectal UC however data from Santiago, Chile and Sao Paulo, Brazil suggest a predominance of colonic CD and pancolonic UC[3,5,7,11,27]. This interesting finding may be associated with differences in genetic polymorphisms or environmental exposures however the potential for confounding due to issues with reporting also need to be considered as colonic CD and pancolonic UC are generally very symptomatic and hence more likely to be reported compared to small bowel CD or more localised UC which is prone to underreporting or misdiagnosis particularly in underprivileged regions. Despite this, it is important to recognise that this high prevalence of colonic CD and pancolonic UC is likely to have important implications for bowel cancer incidence and the requirement of appropriate endoscopic surveillance in such patients within the South American continent.

The hygiene hypothesis postulates that improved hygiene and environmental conditions would reduce the incidence of infections and favour the development of immune-mediated diseases. In this hypothesis, it is thought that exposure to various microbial agents may confer a protective role in promoting immune system maturation by achieving a balance between a pro-inflammatory Th1 response and regulatory T cell tolerance[18,30-32]. This in turn would provide protection against subsequent exposure to antigens and allergens therefore decreasing the likelihood of developing autoimmune conditions such as IBD. Furthermore, the hygiene hypothesis suggests that improved sanitation and reduced exposure to enteric organisms during childhood might lead to inappropriate immunological responses later in life thus increasing the risk of developing IBD[31]. The case-control study conducted in Rio de Janiero by Salgado *et al*[18] was the only identified study from South America which explicitly examined the hygiene hypothesis in CD. The findings demonstrated by

Salgado *et al*[18] suggest that most variables supporting the hygiene hypothesis were associated with CD but were not independent predictors of the diagnosis. Interestingly, this study controversially demonstrated that greater exposure to enteric pathogens was associated with a higher risk for the development of CD[18]. A 94-item survey was the basis of data collection in this study with no serological/microbiological confirmation, therefore a plausible explanation for this unusual finding could be misdiagnosis of intestinal infection at CD onset.

Despite the rigorous methodology followed, this systematic review has several limitations. Firstly, the scarcity of high-quality epidemiological studies on IBD in South America conveyed significant variability between studies which precluded any meta-analysis from being undertaken. Heterogeneity between studies could be explained by methodological limitations, some of which include differences in population characteristics, study designs, access to health care and the variability of diagnostic modalities between countries. Perhaps most significantly, as outlined in Figure 2, the studies included in this review only represented a small geographical area of the South American continent. This limitation has obvious implications for generalisability and further highlights the need for high-quality population-based IBD epidemiological studies from the South American continent. Furthermore, studies were only included in this review if published in the English language which may have resulted in other relevant studies from being overlooked.

A concerted and collaborative South American approach is vital given the future propensity for this region to carry a significant proportion of the worldwide IBD burden. The establishment of central registries within individual countries would be a reasonable first step to overcome the considerable gap of evidence in this region by facilitating the collection of robust and representative data which may help identify aetiological factors and environmental triggers within this unique population. Further education of the primary care sector particularly in identifying risk factors which can be easily and cheaply modified (such as cigarette smoking and breast feeding) as well as differentiating IBD from enteric infection and irritable bowel syndrome will also help decrease morbidity, minimise misdiagnosis and improve patient care. Encouraging utilisation of non-invasive tests such as faecal calprotectin as well as ensuring equitable access to endoscopy will also be beneficial in this regard.

In conclusion, The IBD burden in South America appears to be increasing at a rate greater than other developing regions. Despite this, a paucity of high-quality epidemiological studies continues to exist within this region. The establishment of central registries will help facilitate the collection of robust and representative data to further explore modifiable risk factors and disease phenotypes within this unique population. This information could help facilitate the delivery of high-quality, patient-centred care for South American patients with IBD.

ARTICLE HIGHLIGHTS

Research Background

Inflammatory bowel disease (IBD) refers to a chronic inflammatory disorder of the gastrointestinal tract which is thought to arise from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host. The most common forms of IBD include ulcerative colitis (UC) and Crohn's disease (CD). Whilst previously regarded predominantly as a disease of western nations, the worldwide epidemiology of IBD rapidly changed at the turn of the twenty-first century with studies demonstrating a plateauing of IBD incidence in western nations whilst IBD incidence and prevalence dramatically rose in developing countries from Asia, Africa and Eastern Europe.

Research motivation

Recent efforts have been made to describe IBD in some developing nations within the South American continent however limited collective data is available from this region. Further collective information about IBD within the South American continent will help provide a valuable insight into the emerging epidemic of this disease with the aim of improving the delivery of high-quality patient-centred care within this region.

Research objectives

To summarise the current literature on Inflammatory Bowel Disease in South America and report the incidence, prevalence and disease characteristics of CD and UC within this continent.

Research methods

A systematic review using PRISMA guidelines was undertaken by searching published studies in major international and regional databases between January 1990 and December 2018. Outcomes considered were incidence, prevalence, phenotype, environmental and genetic factors, ethnicity and gender. A pair of independent reviewers screened and reviewed all identified articles.

Research results

One hundred and sixty two citations were initially retrieved with 18 studies included in this systematic review. The majority of included studies were from Brazil (n = 13, 72%). The incidence of UC ranged from 4.3-5.3/100000 person-years whilst the incidence of CD ranged from 0.74-3.5/100000 person-years. Prevalence ranged from 15.0-24.1/100000 inhabitants for UC and from 2.4-14.1/100000 inhabitants for CD. The incidence and prevalence of both UC and CD has increased significantly over the past 20 years. Pancolitis was the most common disease distribution in patients with UC whilst colonic involvement was the most common distribution in CD. People residing in urban areas were at higher risk of developing both CD and UC.

Research conclusions

IBD is an expanding problem within the South American continent with disease burden increasing at a greater rate than other developing regions. Despite this, there remains a paucity of high-queality epidemiological studies from this region. With a total popuation exceeding 400 million, the South American continent is expected to carry a significant proportion of the future global IBD burden.

Research perspectives

This represents the first systematic review to examine the epidemiology of Inflammatory Bowel Disease within South America. Given the current scarcity of high-quality IBD epidemiological studies from this region, a concerted and collaborative South American approach is vital. The establishment of central registries within individual countries would be a reasonable first step to overcome the considerable gap of evidence in this region by facilitating the collection of robust, representative and longitudinal data which may help identify aetiological factors, environmental triggers and modifiable risk factors within this unique population. Further education of the primary care sector, particularly in identifying risk factors which can be easily and cheaply modified (such as cigarette smoking and breast feeding) as well as differentiating IBD from enteric infection and Irritable Bowel Syndrome, will also help decrease morbidity, minimise misdiagnosis and improve patient care. This in turn will help facilitate the delivery of high-quality, patient-centred care for South American patients with IBD.

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