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ORIGINAL ARTICLE

Basic Study

Anti-*Helicobacter pylori* effect of CaG-NANA, a new sialic acid derivative

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Author contributions: Rhee YH performed the majority of experiments, analyzed the data, and wrote the manuscript; Ku HJ and Noh HJ participated in animal experiments; Cho HH, Kim HK and Ahn JC designed and coordinated the research.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Dankook University (IACUC protocol number: DKU-14-036).

Animal care and use statement: The ICR mice (20-22 g) were housed in pathogen free environment and had free access to sterile neutral water and standard mouse feed. Oral administration was performed with conscious animals using sonde appropriate for the animal size.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

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Abstract

AIM

To investigate the bactericidal effects of calcium chelated N-acetylneuraminic acid-glycomacropeptide (CaG-NANA) against *Helicobacter pylori* (*H. pylori*).

METHODS

For manufacture of CaG-NANA, calcium (Ca) was combined with glycomacropeptide (GMP) by chelating, and N-acetylneuraminic acid (NANA) was produced with Ca-GMP substrate by an enzymatic method. The final concentration of each component was 5% Ca, 7% NANA, 85% GMP, and 3% water. For *in vitro* study, various concentrations of CaG-NANA were investigated under the minimal inhibitory concentration (MIC). For *in vivo* study, CaG-NANA was administered orally for 3 wk after *H. pylori* infection. The levels of inflammatory cytokines in blood were analyzed by enzyme-linked immunosorbent assay and eradication of *H. pylori* was assessed by histological observation.

RESULTS

The time-kill curves showed a persistent decrease in cell

numbers, which depended on the dose of CaG-NANA, and MIC of CaG-NANA against *H. pylori* was 0.5% *in vitro*. Histopathologic observation revealed no obvious inflammation or pathologic changes in the gastric mucosa in the CaG-NANA treatment group *in vivo*. The colonization of *H. pylori* was reduced after CaG-NANA treatment. The levels of interleukin (IL)-6, IL-1β, tumor necrosis factor-α, and IL-10 were also decreased by CaG-NANA.

CONCLUSION

CaG-NANA demonstrates effective anti-bactericidal activity against *H. pylori* both *in vitro* and *in vivo*.

Key words: *Helicobacter pylori*; Calcium chealated N-acetylneuraminic acid-glycomacropeptide

Core tip: Calcium chelated N-acetylneuraminic acid-glycomacropeptide demonstrates effective anti-bactericidal activity against *Helicobacter pylori* both *in vitro* and *in vivo*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) has been reported to be associated with many gastrointestinal diseases such as chronic gastritis, peptic ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma[1-3]. Until now, standard triple therapy consisting of a proton pump inhibitor and two broad-spectrum antibiotics, usually amoxicillin, clarithromycin or metronidazole, has been able to achieve eradication[3-5]. However, the Maastricht IV Consensus report recommended the careful choice of the antibiotic combination for treatment according to local *H. pylori* antibiotic resistance patterns due to multidrug resistance of *H. pylori*. For example, the concentrations of antibiotics for treatment of *H. pylori* infection were required at four times when *H. pylori* had local clarithromycin resistance[6]. Since antibiotic abuse had side effects and allowed development of resistance to antibiotics, alternative strategies have been proposed to counteract *H. pylori* infection. As an alternative approach, preservation of mucus from *H. pylori* attachment is emphasized because *H. pylori* infection disrupted the epithelial barrier which resulted in inflammation or cancer[7]. For example, dietary inhibitors such as lacto-oligosaccharide has been suggested as a solution for *H. pylori* infections[8]. Although oligosaccharides specific for the *H. pylori* lectins may potentially act as inhibitors of adhesion to mucus, their production in commercial amounts as anti-adhesion therapeutic agents is still a problem[9,10]. Here, we introduce a new N-acetylneuraminic acid (NANA) combined glycomacropeptide (GMP) which was made from milk serum hydrolysis protein powder as an anti-*H. pylori* agent. Wadström *et al*[11] reported that NANA derivative had an adhesion potential to *H. pylori* lectins and Hirmo *et al*[12] reported that milk glycoprotein had an anti-*H. pylori* effect. We designed a new material with NANA, GMP, and calcium (CaG-NANA), and anti-*H. pylori* activities of CaG-NANA were investigated both *in vitro* and *in vivo*.

MATERIALS AND METHODS

H. pylori strain and experimental animals

H. pylori strain (SS1-passed-5) was kindly provided by Helicobacter pylori Korean Type Culture Collection (HpKTCC, Jinju, South Korea) and grown in 1.5% agar added Brucella broth with 10% horse serum (No. CM0169; OXOID, Waltham, MA, United States). The pathogen-free (SPF) male ICR mice (20-22 g) were purchased from Orient Bio (Daejeon, South Korea). All the animals were housed in an SPF environment and had free access to sterile neutral water and standard mouse feed. The experimental procedures in this study were approved by the Experimental Animal Ethics Committee of Dankook University, South Korea (No. DKU14-036).

Supply of CaG-NANA

CaG-NANA and each component of CaG-NANA were provided by MediNutrol (Kwangju, South Korea). As shown in Figure 1A, CaG-NANA was manufactured from calcium (Ca), GMP, and NANA by chelating and enzyme methods where the component concentrations were 5%, 85%, and 7%, respectively. For comparing each produced compound, we wrote abbreviated form as standard NANA (S-NANA), GMP linked NANA (G-NANA), and calcium chelated G-NANA (CaG-NANA).

High performance liquid chromatography for confirmation of NANA

For confirmation of NANA component, we analyzed CaG-NANA using high performance liquid chromatography (HPLC) method. HPLC analysis was performed using Agilent 1260 model equipped with a pump (G1311C), an auto sampler (G1329B), a column (G1316A), and a ultraviolet detector (G1314F), which was purchased from Agilent (Santa Clara, CA, United States). The condition of analysis was described in Table 1.

Culture and collection of *H. pylori*

H. pylori was incubated in Brucella medium contained the selective supplements (No. SR0083; OXOID, Waltham, MA, United States) under microaerobic environment (15% CO_2, 5% O_2, 80% N_2) at 37 ℃ for 72 h. The bacterial colonies were collected and identified

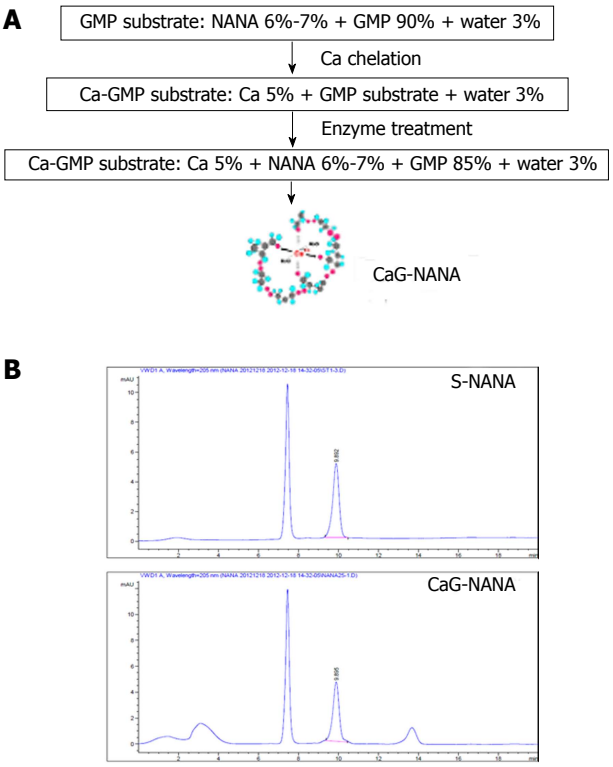

Figure 1 Confirmation of N-acetylneuraminic acid. A: The scheme of CaG-NANA manufacture; B: HPLC analysis of NANA content in CaG-NANA. The condition of HPLC analysis is described in Table 1. GMP: Glycomacropeptide; HPLC: High performance liquid chromatography; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid; S-NANA: Standard N-acetylneuraminic acid.

with a Pronto-Dry infection kit (Kokab Enterprise, Karachi, Pakistan) for bactericidal tests *in vitro*.

Inhibitory effect of CaG-NANA on H. pylori in vitro

The inhibitory activity of CaG-NANA against the growth of *H. pylori* was assessed using an agar dilution method. Briefly, 0.1%, 0.25%, or 0.5% of CaG-NANA and its components were added to the Brucella agar. The non-drug agar served as a negative control. Agar plates were inoculated with *H. pylori* at serial concentrations of 1×10^8, 1×10^7, and 1×10^6 colony forming units (CFU)/mL and cultured for 72 h. The minimal inhibitory concentration (MIC) was defined as the minimal concentration of CaG-NANA required for complete inhibition of *H. pylori* growth. The colonies were counted using image J (https://imagej.nih.gov/ij/) after 72 h incubation and the average number was calculated. Bactericidal activity was evaluated using time-kill curves with 0.5, 1.0 and 2.0 × MIC of CaG-NANA compared with the blank controls.

Experimental design in vivo

Mice (n = 40) were randomly divided into four groups: Blank control, *H. pylori* infected control, antibiotic treatment, and CaG-NANA treatment. *H. pylori* (1×10^9 /mL) was administered by gastric intubation to mice three times in a week except the blank control, and *H. pylori* infection was detected with gastric irrigation from randomly selected mice using an *H. pylori* detection test kit which was purchased from Pronto Dry (Brignais, France; Supple 1A). The antibiotic treatment was performed with a suspension of amoxicillin (12.33 mg/kg), metronidazole (164.40 mg/kg), and clarithromycin (205.54 mg/kg) which were equivalent to clinical administration (Dankook University hospital, Korea). Treatments with CaG-NANA and each component including NANA, S-NANA, and G-NANA were performed by oral administration every day for 3 wk, and the experimental design is shown in Figure 2A.

Table 1 High performance liquid chromatography analysis condition

Detector	UV detector
Wavelength	205 nm
Column	Aminex HPX-87H Ion Exclusion column (300 mm × 7.8 mm, 9 μm)
Mobile phase	10 mmol/L H_2SO_4 in water (isocratic)
Running time	20 min
Flow rate	0.5 mL/min
Injection volume	10 mL
Temperature	40 ℃

Histological observation

The animals were deprived of feed but allowed free access to water for 24 h before sacrificed. At the end of sacrifice, blood was collected from mouse and gastric tissues were removed and fixed in 10% formalin. After fixation, tissues were processed and embedded in paraffin. The paraffin blocks were cut into 4 μm sections and stained with Harris' hematoxylin and eosin for histological observation under a microscope (BX51, Olympus, Miami, FL, United States).

Enzyme-linked immunosorbent assay

The expression levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-10 in blood serum were determined using enzyme-linked immunosorbent assay (ELISA). Mouse ELISA kits were purchased from R&D Systems (Minneapolis, MN, United States). The assays were performed according to the manufacturer's instructions and repeated in triplicate.

Statistical analysis

Eradication rates were compared among groups by one-way analysis of variance (Kruskal-Wallis) and Dunn's multiple comparison test (GraphPad Software Inc., La Jolla, CA, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Confirmation of NANA

As shown Figure 1B, the NANA component of CaG-NANA showed the same purity as standard NANA.

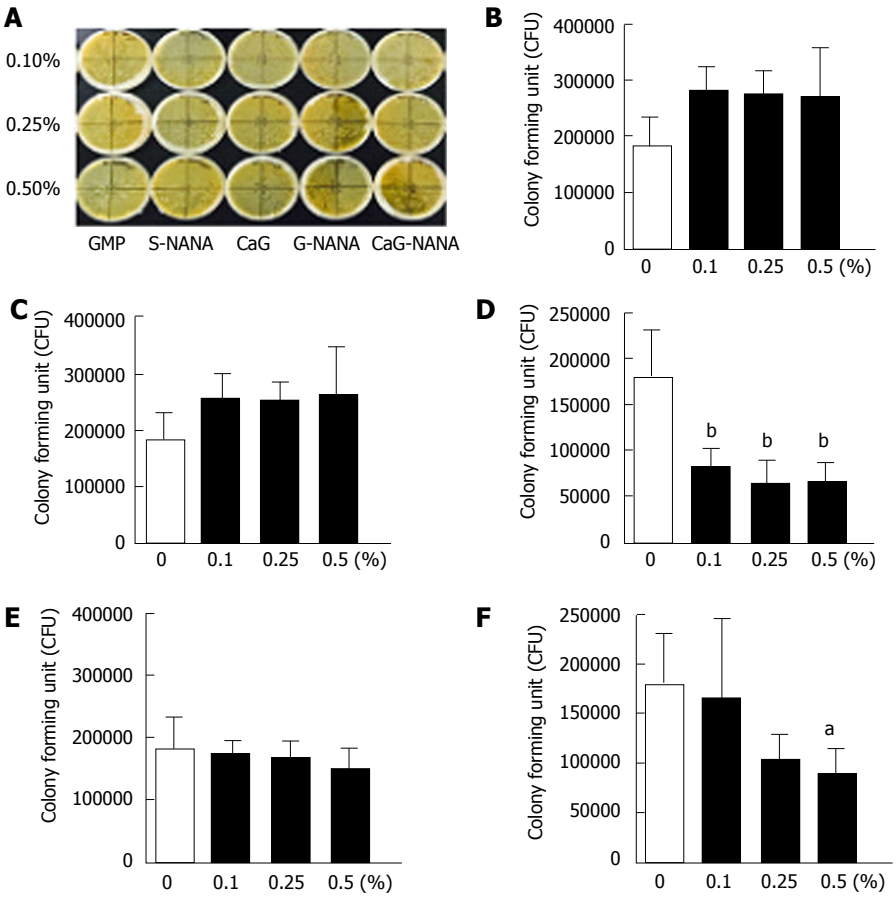

Figure 2 Inhibitory effect of calcium-glycomacropeptide-N-acetylneuraminic acid on *Helicobacter pylori in vitro*. Agar plates were inoculated with *Helicobacter pylori* (*H. pylori*) at serial concentrations of 1 × 10^8, 1 × 10^7, and 1 × 10^6 CFU/mL and cultured for 72 h. The minimal inhibitory concentration (MIC) was defined as the minimal concentration of materials required for complete inhibition of *H. pylori* growth. Bactericidal activity was evaluated using time-kill curves with 0.5, 1.0 and 2.0 × MIC of CaG-NANA compared with blank controls. All experiments were performed three times and significance was set at $^aP < 0.1$ and $^bP < 0.05$. A: Picture of colony forming unit assay; B: GMP; C: CaG; D: S-NANA; E: G-NANA; F: CaG-NANA. GMP: Glycomacropeptide; CaG: Calcium-glycomacropeptide; S-NANA: Ststandard N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

Inhibitory effect of CaG-NANA on H. pylori in vitro

The bactericidal effects of CaG-NANA and each component against *H. pylori* were assessed *in vitro*. We described previously that CaG-NANA is composed of NANA, GMP and calcium, thus we tested the anti-bacterial effects of every component used in CaG-NANA synthesis. As shown in Figure 1, S-NANA, G-NANA and CaG-NANA had an anti-bacterial effect (Figure 1D, E and F) whereas GMP and GMP with calcium (CaG) had no activity in the colony forming assay (Figure 1B and C). This result indicated that only NANA included compound had anti-bacterial activity.

Inhibitory effect of CaG-NANA on H. pylori in vivo

For the *in vivo* study, we divided mice into four groups and 1 × 10^9/mL CFU of *H. pylori* was administered into mice three times for one week except the negative group. *H. pylori* infection was confirmed by gastric irrigation using an *H. pylori* detection test kit (Pronto Dry). After confirmation, treatments with antibiotics, CaG-NANA and its components were orally administered every day for 3 wk. The doses of antibiotics were described in the "Material and Methods" section (Figure 3A). The doses of CaG-NANA and its components were fixed at 0.5% (v/w). We followed the mouse weight after treatment. As shown in Figure 3B, mouse weight was decreased in the S-NANA and *H. pylori* positive control groups. From this result, S-NANA was considered to have toxicity at 0.5% of concentration.

Next, we observed the gastric mucus layer of mice. The majority of the *H. pylori* population reside in the mucus which binds the organisms *via* specific interactions such as pathogen adherence. Normal gastric mucosa has a uniform surface epithelium (Figure 4A), whereas *H. pylori* infected gastric mucosa has an irregular outline of the epithelium layer along with neutrophil and macrophage infiltrations (Figure 4B). In addition, bacterial attachment is mediated by outer membrane adhesions that bind to glycoconjugates present in the gastric mucus layer, lining the surface epithelium of the gastric mucosa in the *H. pylori* infected group. As shown in Figure 4C, the antibiotic treatment group had decreased infiltration of macrophages and adhesion of *H. pylori* colonization, however, the destruction of surface epithelium layers was also observed. Meanwhile, every NANA including group showed uniformed surface epithelium layers. Interestingly, many macrophages and neutrophils were observed in the S-NANA treatment

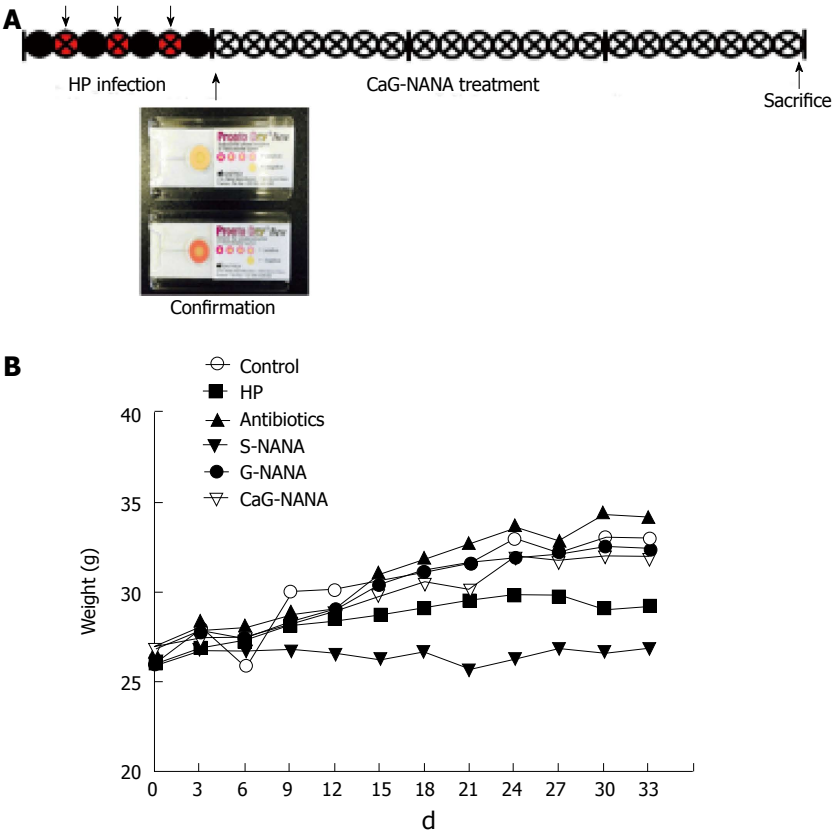

Figure 3 Inhibitory effect of calcium-glycomacropeptide-N-acetylneuraminic acid on *Helicobacter pylori in vivo*. Antibiotic doses are described in the "materials and methods" section. A: Schematic design of *in vivo* study; B: Weight changes of mice. HP: Helicobacter pyroli; S-NANA: Standard N-acetyln-euraminic acid; G-NANA: Glycomacropeptide-N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

group (Figure 4D). The damage to surface epithelium in the antibiotics treatment group and inflammation observed in the S-NANA treatment group might be related to mouse weight loses. Both the G-NANA and CaG-NANA treatment groups showed the detachment of *H. pylori* colonization without any damage to surface epithelium or inflammation (Figure 4E and F).

We also assessed the levels of IL-1β, IL-6, TNF-α, and IL-10 in blood by ELISA. PBS was used as a control because every compound was dissolved in PBS. Both of S-NANA and CaG-NANA showed a potent ability to decrease inflammatory cytokines. The level of IL-1β was reduced from 7.6 ng/mL to 0.8 ng/mL after S-NANA treatment and 2.36 ng/mL after CaG-NANA treatment (Figure 5A). The level of IL-6 was remarkedly decreased from 113.4 ng/mL to 33.5 ng/mL after S-NANA treatment and 1.26 ng/mL after CaG-NANA treatment (Figure 5B). The level of TNF-α was also reduced to 8.21 ng/mL after S-NANA treatment and 1.44 ng/mL after CaG-NANA treatment (Figure 5C). Meanwhile, the level of IL-10 was reduced only in the CaG-NANA treatment group from 7.62 ng/mL to 6.3 ng/mL (Figure 5D).

DISCUSSION

The anti-*H. pylori* effects of antibiotic formulas have been investigated, but the findings are limited by varying drug quality and sources, as well as the numerous and complicated components of formulas. Thus, it has been suggested that dietary inhibitors may be a solution for certain infections as an alternative approach[8]. Eradication of *H. pylori* has remained difficult for reasons that lie in the biology and environment of the organism. *H. pylori* populations colonize epithelial cells that line the antrum of the stomach and survive in the acidic environment[13]. Most anti-microbial agents are poorly secreted in the gastric mucosa because of the stomach environment[14], and the residues expressed on *H. pylori* enable specific binding to the mucus layers. Thus, CaG-NANA was excogitated to overcome the acidic environment of the stomach and have bactericidal activity by interrupting glycol-conjugation of *H. pylori* on the mucosa.

CaG-NANA, a compound of N-acetylneuraminic acid with calcium combined glycomacropeptide, demonstrated an anti-bacterial effect against *H. pylori*. We manufactured CaG-NANA as a dietary inhibitor against *H. pylori*; NANA was demonstrated as an *H. pylori* adhesion blocker[9,15,16], glycomacropeptide was used for modulating the acidic phase of the stomach, and calcium was inserted for improvement as functional food. NANA content in CaG-NANA was evaluated compared to S-NANA by HPLC, which confirmed its content and purity (Figure 1B). CaG-NANA at concentrations > 0.25% regulated the population of *H. pylori in vitro*. S-NANA also decreased the population of *H. pylori in vitro* (Figure 2D); however, S-NANA resulted in a severe weight loss *in vivo* (Figure 3B), which demonstrated that S-NANA treatment only had negative utility. Meanwhile, G-NANA and CaG-NANA had bactericidal and anti-adhesion effects on glyco-conjugation of the mucosa without toxicity. Bacterial attachment is mediated by outer membrane adhesions that bind to glycoconjugates present in the gastric mucus layer and lining the surface epithelium of

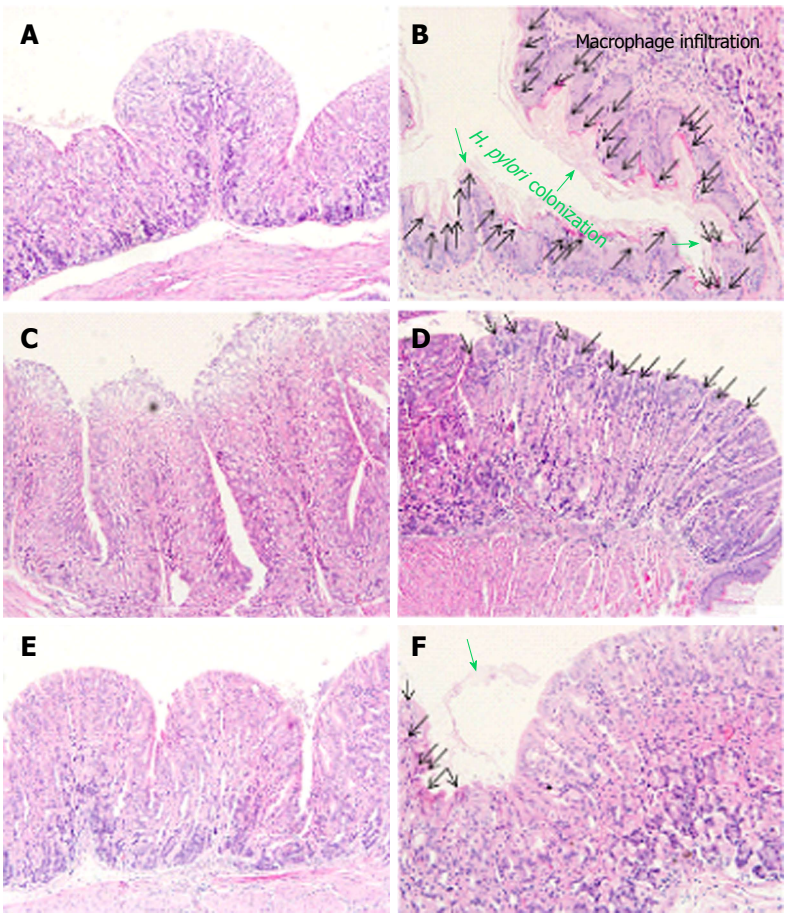

Figure 4 Histology of the gastric mucosa. Mouse stomachs were removed and underwent hematoxylin and eosin staining. *H. pylori* colonization and macrophage infiltration were observed under a light microscope. A: Normal group; B: *H. pylori* infected group; C: Antibiotics treated group; D: S-NANA treated group; E: G-NANA treated group; F: CaG-NANA treated group. S-NANA: Standard N-acetylneuraminic acid; G-NANA: Glycomacropeptide-N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

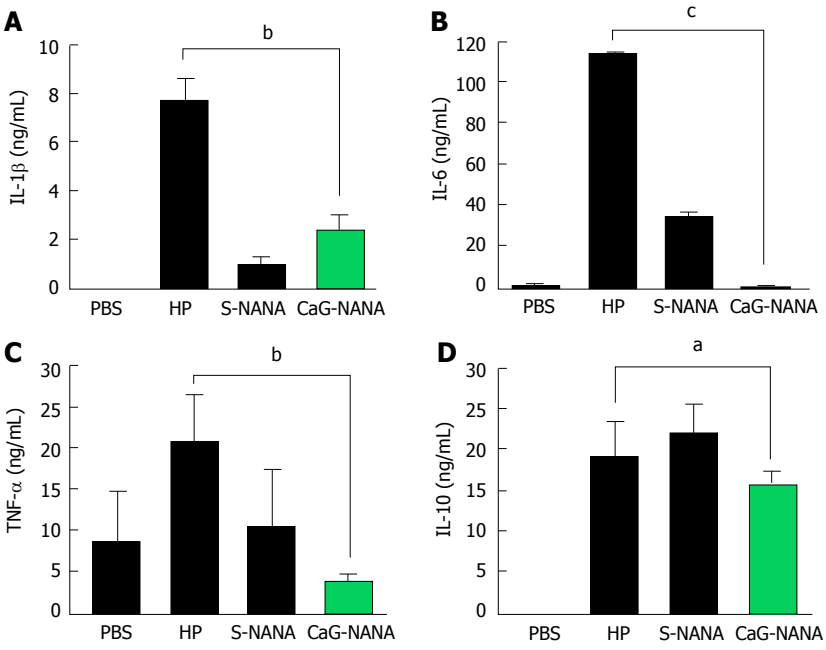

Figure 5 Expression levels of interleukine-1β, interleukine-6, tumor necrosis factor-α, and interleukine-10 in blood serum determined by enzyme-linked immunosorbent assay. The assays were performed according to the manufacturer's instructions and all experiments were repeated in triplicate. Statistical analysis was performed using Dunn's multiple comparison test with significance set at $^{a}P < 0.1$ and $^{b}P < 0.05$, $^{c}P < 0.01$. A: IL-1β; B: IL-6; C: TNF-α; D: IL-10. IL: Interleukine; TNF: Tumor necrosis factor; S-NANA: Standard N-acetylneuraminic acid; G-NANA: Glycomacropeptide-N-acetylneuraminic acid; CaG-NANA: Calcium-glycomacropeptide-N-acetylneuraminic acid.

the gastric mucosa[17].

H. pylori is also known to induce inflammatory response that includes the up-regulation of pro-inflammatory cytokines. CaG-NANA exerted a down-regulatory effect on pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IL-10. Reduction of pro-inflammatory cytokines induced by *H. pylori* is responsible for the recruitment of macrophages and neutrophils in the lamina propria. CaG-NANA exerted an antagonistic effect against *H. pylori*, which is an anti-microbial effect *via* different mechanisms from those of antibiotics.

This study suggests that it is possible to decrease the number of *H. pylori* or its activation in the stomach through a regular ingestion of N-acetylneuraminic acid containing glycomacropeptide as dietary products. Moreover, CaG-NANA is a complex compound which can be manufactured in large scale at a low cost under good manufacturing practice (GMP). In conclusion, the anti-*H. pylori* effects of CaG-NANA were confirmed both *in vitro* and *in vivo*, which provided experimental support for future human trials.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) has been reported to be associated with many gastrointestinal diseases such as chronic gastritis, peptic ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma. Until now, standard triple therapy consisting of a proton pump inhibitor and two broad-spectrum antibiotics, usually amoxicillin, clarithromycin or metronidazole, has been able to achieve eradication.

Research frontiers

The authors introduce a new N-acetylneuraminic acid (NANA) combined glycomacropeptide (GMP) which was made from milk serum hydrolysis protein powder as an anti-*H. pylori* agent. Wadstorm *et al* reported that NANA derivative had an adhesion potential to *H. pylori* lectins and Hirmo *et al* reported that milk glycoprotein had an anti-*H. pylori* effect. The authors designed a new material with NANA, GMP, and calcium (CaG-NANA), and anti-*H. pylori* activities of CaG-NANA were investigated both *in vitro* and *in vivo*.

Innovations and breakthroughs

The anti-*H. pylori* effects of CaG-NANA were confirmed both *in vitro* and *in vivo*, which provided experimental support for future human trials.

Applications

CaG-NANA is a complex compound which can be manufactured in a large scale at a low cost under GMP.

Peer-review

In the present paper, entitled "Anti-*Helicobacter pylori* effect of CaG-NANA, a new sialic acid derivative", Rhee *H et al* have investigated the effect of a compound named Cag-NANA in an animal model of *H. pylori*-associated gastritis and, *in vitro*, in bacterial cultures.

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ORIGINAL ARTICLE

Retrospective Study

Microscopic colitis in patients with mild duodenal damage: A new clinical and pathological entity ("lymphocytic enterocolitis")?

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Author contributions: Bonagura GA and Ribaldone DG equally contributed to this paper; Bonagura GA, Ribaldone DG, Saracco GM, Astegiano M and Pellicano R designed the research; Bonagura GA, Ribaldone DG, Fagoonee S and Sapone N performed the research; Fagoonee S and Caviglia GP analyzed the data; Bonagura GA, Ribaldone DG, Astegiano M and Pellicano R wrote the paper.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Molinette Hospital.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous data and it was performed several years after the consultation (retrospective).

Conflict-of-interest statement: None to declare.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Abstract

AIM

To evaluate the potential association between mild duodenal damage and microscopic colitis (MC).

METHODS

We retrospectively included 105 consecutive patients with type I Marsh-Oberhuber duodenal damage and negativity for immunoglobulin A anti-endomysium and anti-tissue transglutaminase. The following parameters were analyzed: Sex, age at execution of esophago-gastroduodenoscopy, duodenal damage, and number of intraepithelial lymphocytes at biopsies, prevalence

of *Helicobacter pylori* infection, age at execution of colonoscopy, macroscopic and microscopic features of colonoscopy, family history of gastrointestinal and autoimmune diseases, smoking habits, biochemical parameters of inflammation and autoimmunity, use of proton pump inhibitors or nonsteroidal anti-inflammatory drugs, adverse reactions to drugs or foods, pathologies known to be associated with celiac disease or MC, living on a gluten-free diet or on a gluten-low diet for at least 1 mo.

RESULTS

Colonoscopy was performed in 59 patients, but only in 48 of them biopsies were taken in the entire colon. Considering the latter cohort, the diagnosis of MC was met in 25 (52.1%) patients while in 18 patients other pathologic findings were reported: 13 (27%) cases of nonspecific inflammatory bowel disease, 2 (4.2%) cases of Crohn's disease, 2 (4.2%) cases of eosinophilic gastroenteritis, and 1 (2.1%) case of autoimmune enteritis. Five (10.4%) patients had a normal colonoscopic result. Matching the groups by age, and considering only patients who underwent colonoscopy (42.7 ± 15.5 years) *vs* those who did not undergo colonoscopy (36.9 ± 10.6 years), a statistical difference was found (P = 0.039). Focusing on symptoms, diarrhea was statistically more prevalent in MC group than in patients who did not undergo colonoscopy (P = 0.03).

CONCLUSION

Mild duodenal damage is associated with MC in more than half of the cases. This association supports the hypothesis of a link between these two entities.

Key words: Autoimmune diseases; Celiac disease; *Helicobacter pylori*; Intraepithelial lymphocytes; Lymphocytic colitis; Lymphocytic enterocolitis; Microscopic colitis

Core tip: Scarce information is available on patients with symptoms suggestive for celiac disease but with negative serologic tests and mild duodenal damage (type I Marsh-Oberhuber classification). Our data show that mild duodenal damage is associated with microscopic colitis in more than the half of the investigated cases. This association may support the hypothesis of a new clinical and pathological entity, the "lymphocytic enterocolitis".

Bonagura GA, Ribaldone DG, Fagoonee S, Sapone N, Caviglia GP, Saracco GM, Astegiano M, Pellicano R. Microscopic colitis in patients with mild duodenal damage: A new clinical and pathological entity ("lymphocytic enterocolitis")? *World J Gastrointest Pathophysiol* 2016; 7(4): 307-313 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i4/307.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i4.307

INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disease characterized by a pathological reaction against gluten proteins[1]. Currently, the prevalence of CD in the general population is proximally 1%, with a ratio between diagnosed and undiagnosed cases of about 1:7[2,3]. CD presents often signs and symptoms such as chronic diarrhea, bloating, abdominal pain and malabsorption[4]. However, in a substantial number of cases, CD can manifest only extra-intestinal symptoms or signs, and it can be associated with autoimmune pathologies, as autoimmune thyroiditis, type I diabetes mellitus and rheumatoid arthritis[5,6]. The diagnosis of CD is based on the finding of positive antibody tests (anti-endomysium and anti-tissue transglutaminase), confirmed by biopsies taken during esophagogastroduodenoscopy (EGD) that reveal the characteristic duodenal damage. The Marsh-Oberhuber classification is usually used to grade the severity of duodenal lesions, with the type III representative of CD[7]. The search for human leukocyte antigen (HLA) haplotypes DQ2 and DQ8, due to its high negative predictive value, is used to exclude CD[8]. Nevertheless, there are patients with suggestive symptoms of CD, mild duodenal damage [*i.e.*, an increase of intraepithelial lymphocytes (IEL)] defined type I, according to Marsh-Oberhuber classification, and negative antibody tests. This clinical condition, that does not conform with the diagnosis of CD, needs to be investigated for other causes[9].

Microscopic colitis (MC) is a chronic inflammatory bowel disease, distinct in lymphocytic colitis (LC)[10] and collagenous colitis (CC)[11]. The diagnosis of MC is obtained by multiple colonic mucosal biopsies taken during colonoscopy[12]. Typically, in CC, the histological feature is a thickening of the subepithelial collagen layer beneath the basal membrane, of more than 7-10 μm (0-3 μm in the normal colon)[13]. The histological feature of LC is the presence of more than 20 IEL/100 surface epithelial cells (< 5 IEL/100 in the normal colon)[14]. Paucicellular LC is a term used when the number of IEL is comprised between 5 IEL/100 and 20/100 surface epithelial cells. In MC, IEL are T-Lymphocyte $CD3^+$ and $CD8^+$, similar to those described in case of type I Marsh-Oberhuber lesions. Previously considered rare, MC is now a relatively common cause of chronic watery nonbloody diarrhea, especially in the elderly[15]. Both LC and CC are associated with autoimmune diseases and allergy[16]. Finally, it has been shown that patients with MC have an increased rate of HLA-DQ2 and HLA-DQ8 positivity[17], even if this association is less strict than with CD.

Although some authors reported an association between MC and type I Marsh-Oberhuber duodenal damage[18-23], the interpretation of this finding is poorly described. Nevertheless, there are few studies[24] that searched for the inverse association.

The aim of this study was to evaluate, for the first time, the association between type I Marsh-Oberhuber

duodenal damage and MC, arguing for the existence of a possible "microscopic enterocolitis"[25,26].

MATERIALS AND METHODS

We retrospectively included 105 (86 females, mean age 40.1 ± 13.7) consecutive patients with type Ⅰ Marsh-Oberhuber duodenal damage and negativity for anti-endomysium (EmA) and anti-tissue transglutaminase (tTG) immunoglobulin (Ig)A antibodies. No sign of Whipple disease were reported in duodenal biopsies. Patients affected by small bowel bacterial overgrowth were excluded from the analysis. The analysis included patients observed in the period 1 January 2003-31 December 2013 in the outpatients clinic of the Unit of Gastroenterology and Hepatology, Molinette Hospital, Turin, Italy.

In 5 cases of IgA deficiency, the genetic assessment (HLA-DQ2/DQ8) was performed: In 3, the result was negative while in the remaining 2 HLA-DQ2 positivity was found.

The following parameters were analyzed: Sex, age at execution of EGD, duodenal damage with number of IEL at biopsies, age at execution of colonoscopy, macroscopic and microscopic features of colonoscopy, family history of gastrointestinal and autoimmune diseases, smoking habits, dosage of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and anti-nuclear antibody (ANA), use of proton pump inhibitors (PPIs) or nonsteroidal anti-inflammatory drugs (NSAIDs)[27], adverse reactions to drugs or foods, pathologies associated with CD or MC, living on a gluten-free diet or on a gluten-low diet for at least 1 mo. Data on the prevalence of watery diarrhea, constipation, epigastric pain, abdominal pain, weight loss, nausea and/or vomiting, bloating, and asthenia were collected. Malabsorption was defined as the presence of at least one of these elements: Hemoglobin (Hb) < 12 g/L and low levels of serum iron or folate or vitamin B12; hypoalbuminemia; weight loss > 10% without hypocaloric diet. *Helicobacter pylori* (*H. pylori*) infection was investigated by urea breath test and gastric biopsies. The previous eradication treatment of this infection, if any, was reported.

The pharmacological anamnesis for assumption of prednisone, mesalamine, salazopyrin, budesonide, and antibiotics (rifaximin, ciprofloxacin, metronidazole) was conducted. Patients who took serotonin reuptake inhibitors or antiplatelets were excluded from the analysis.

Statistical analysis

For parametric data, we initially used the "normal probability plot", to value a normal data distribution; in case of positive return, the Student T test to match the two subgroups was used.

For non-parametric data, the subgroups were matched with the Yates' χ^2 test or with the Fisher's exact test if data were $\leqslant$ 5.

Confidence interval (CI) was set at 95%, with the statistical significance set at P value < 0.05. All statistical analyses were performed using MedCalc software (MedCalc Software, version 9.2.1.0).

RESULTS

Overall, colonoscopy was performed in 59 patients, but in only 48 cases biopsies were taken along the entire colon. In the remaining 11 cases, biopsies were not taken or taken only in the left colon. The histological findings permitted to divide the cohort into two groups: That including 25 patients with diagnosis of MC and that including 23 patients without MC (Figure 1).

Matching by age, patients who underwent colonoscopy (42.7 ± 15.5 years) *vs* those who did not undergo colonoscopy (36.9 ± 10.6 years), a statistical difference was found (P = 0.039). On the contrary, there was no significant difference between the group of patients who did not undergo colonoscopy (36.9 ± 10.6 years) *vs* the group who underwent colonoscopy and executed multiple biopsies (40.4 ± 13.7 years) (P = 0.186). Considering the symptoms, there were no statistical differences between patients who did not undergo colonoscopy *vs* those who underwent colonoscopy and executed multiple biopsies (P = 0.09 and P = 0.14 for epigastric pain and diarrhea, respectively). Diarrhea was statistically more prevalent in MC group than in patients who did not undergo colonoscopy (P = 0.03). Patients who did not undergo colonoscopy *vs* those who underwent colonoscopy and executed multiple biopsies had not statistical differences when comparing the heterodimers HLA-DQ2 and HLA-DQ8 (P = 0.19).

Among patients who underwent colonoscopy, an inflammatory pattern was found in 89.6% of cases. Focusing on the 25 patients with MC, the females to males ratio resulted 5:1 and the mean age was 40 ± 16.3 years. The diagnosis was LC in 13 cases, paucicellular LC in 9, CC in 2, and undefined MC in the remaining patient. The average duodenal IEL were 41.6/100 epithelial cells and colonic IELs were 25.4/100 epithelial cells. Watery diarrhea was present in 17/25 (68%) patients, abdominal pain in 16/25 (64%), weight loss in 11/25 (44%), nausea or vomiting in 7/25 (28%), epigastric pain in 6/25 (24%), asthenia in 5/25 (20%), bloating in 4/25 (16%), and gastroesophageal reflux disease (GERD) in 2/25 (8%). A family history of Crohn's disease, thyroiditis, rheumatoid arthritis or spondylitis, was present in 1/25 (4%) patient for each one. Regarding smoking habits, 19/25 (76%) patients were non-smokers while the remaining 6 (24%) were smokers. ANA test resulted positive in 4/25 (16%) patients, ESR increased in 2/25 (8%), and CRP in 2/25 (8%). Four out of twenty-five (16%) patients had a positive history of PPIs use, and 1/25 (4%) of NSAIDs use. Autoimmune thyroiditis was diagnosed in 4/25 (16%) patients, asthma in 3/25 (12%), rheumatoid arthritis in 3/25 (12%). Anamnesis of adverse reactions to drugs or foods resulted in 10/25 (40%) patients.

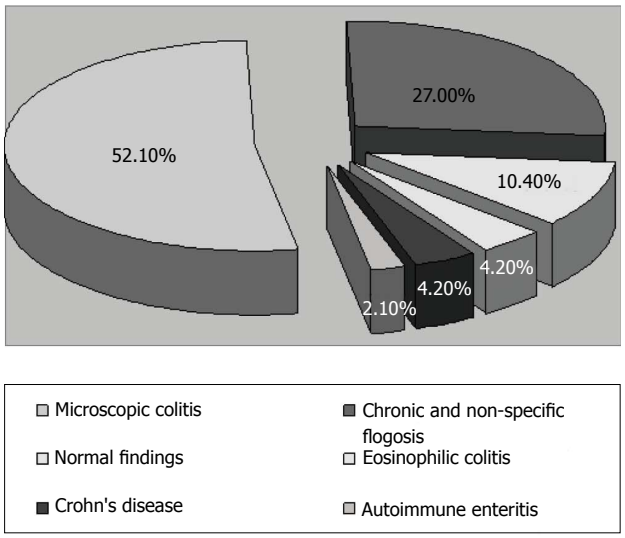

Figure 1 Microscopic findings at colonoscopy (48 cases).

Table 1 Main clinical and laboratory parameters of enrolled patients

	Patients with MC	Patients without MC	*P* value
Watery diarrhea	17/25 (68%)	10/23 (43%)	0.08
Abdominal pain	16/25 (64%)	10/23 (43%)	0.15
Weight loss	11/25 (44%)	3/23 (13%)	0.01
Nausea/vomiting	7/25 (28%)	4/23 (17%)	0.29
Epigastric pain	6/25 (24%)	5/23 (21%)	0.85
Asthenia	5/25 (20%)	4/23 (17%)	0.55
Bloating	4/25 (16%)	5/23 (21%)	0.44
GERD	2/25 (8%)	4/23 (17%)	0.33
Constipation	0/25 (0%)	4/23 (17%)	0.04
History of allergy	10/25 (40%)	10/23 (43%)	0.40
PPIs use	4/25 (16%)	6/23 (26%)	0.44
NSAIDs use	1/25 (4%)	0/23 (0%)	0.34
ANA positivity	4/25 (16%)	8/23 (34%)	0.46
ESR increased	2/25 (8%)	6/23 (26%)	0.13
CRP increased	2/25 (8%)	5/23 (21%)	0.21
Helicobacter pylori infection	6/25 (24%)	8/23 (34%)	0.61

MC: Microscopic colitis; GERD: Gastroesophageal reflux disease; PPIs: Proton pump inhibitors; NSAIDs: Nonsteroidal anti-inflammatory drugs; ANA: Anti-nuclear antibody; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein.

Thirteen patients (52%) had HLA-DQ2 positivity, 8 (32%) HLA-DQ2/DQ8 negativity, and 4 (16%) HLA-DQ8 positivity. Regarding *H. pylori* infection, 19/25 (76%) had negativity *ab initio* while 4 out of 6 with positivity (66.6%), eradicated the infection after antibiotic treatment. None of the tested patients had positivity at coproculture or at parasitological fecal test (6 and 2 cases, respectively). Fourteen (56%) patients undertook a gluten-free diet for at least 1 mo with a clinical improvement in 3/14 (21.4%). Malabsorption was observed in 12 (48%) patients.

Among patients without MC, the female to male ratio resulted 3.8:1 and the mean age was 40.5 ± 13.7 years. The diagnosis was of chronic and non-specific inflammation in 13 (56.5%) cases, there was a normal finding in 5 (21.7%), eosinophilic colitis in 2 (8.6%), Crohn's disease in 2 (8.6%), and autoimmune enteritis in the last one (4.3%). The average duodenal IEL resulted 42.1/100 epithelial cells. Based on the available data, a family history of Crohn's disease, rheumatoid arthritis or spondylitis was reported in one out of 23 (4.3%) patients for each disease. Regarding smoking habits, 15/23 (65.2%) patients were non-smokers while the remaining 8 (34.8%) were smokers. Abdominal pain was present in 10/23 (43.4%) patients, watery diarrhea in 10/23 (43.4%), epigastric pain in 5/23 (21.7%), bloating in 5/23 (21.7%), asthenia in 4/23 (17.3%), nausea or vomiting in 4/23 (17.3%), GERD in 4/23 (17.3%), constipation in 4/23 (17.3%), and weight loss in 3/23 (13.0%). ANA test resulted positive in 8/23 (34.8%) patients, ESR and CPR was increased in 6/23 (26.1%) and 5/23 (21.7%), respectively. A history of PPIs or NSAIDs use was reported in 6/23 (26.1%) and no patient, respectively. Autoimmune thyroiditis was reported in 3/23 (13%) of the patients, asthma in 2/23 (8.6%), rheumatoid arthritis in 2/23 (8.6%), systemic erythematosus lupus (SLE), autoimmune hepatitis and multiple autoimmune diseases in 1/23 (4.3%) for each one. Adverse reactions to drugs or foods resulted in 10/23 (43.4%) patients. Ten (43.4%) patients had HLA-DQ2/DQ8 negativity, 9 (39.1%) had HLA-DQ2 positivity, 3 (13%) HLA-DQ8 positivity, and one (4.3%) had HLA-DQ2/DQ8 positivity. Regarding *H. pylori* infection, 15/23 (65.2%) of the tested patients were negative *ab initio*, while 8/23 (34.8%) were positive. Of the latter group, 5 out of 8 (62.5%) eradicated the infection after antibiotic treatment. None of the tested patients had positivity at coproculture or at parasitological fecal test (4 and 1 case, respectively).

Comparing patients with MC *vs* those without MC (Table 1), the only variables that had a statistical difference were weight loss ($P = 0.01$), more frequent in case of MC, and constipation ($P = 0.04$) more frequent in absence of MC. Diarrhea ($P = 0.08$), abdominal pain ($P = 0.15$), epigastric pain ($P = 0.85$), GERD ($P = 0.33$), autoimmune thyroiditis ($P = 0.79$), smoking habits ($P = 0.66$), asthma ($P = 0.72$), rheumatoid arthritis ($P = 0.72$), autoimmune hepatitis ($P = 0.31$), multiple autoimmune diseases ($P = 0.35$), HLA-DQ2 positivity ($P = 0.51$), HLA-DQ8 positivity ($P = 0.79$) did not reach statistical significance.

Among patients suffering from MC, budesonide was used in 14 patients, of whom 13 (92.9%) responded to therapy; 8 patients used mesalamine, of them 4 (50%) responded to therapy; 4 patients used salazopyrin, with response in 2 (50%); 1 patient used prednisone, with response. No difference about the response to therapy resulted from the comparison between budesonide and mesalamine ($P = 0.27$), budesonide and salazopyrin ($P = 0.41$), budesonide and prednisone ($P = 0.94$). Among patients without MC, 7 used budesonide and 6/7 (85.7%) responded to therapy; 4 patients used prednisone without response; 6 patients used mesalamine with response in 3 (50%).

DISCUSSION

In this study, we found a strong association between type Ⅰ Marsh-Oberhuber duodenal damage and MC, mainly LC. More than half (52.1%) of the patients who underwent colonoscopy with multiple biopsies had MC. This percentage is significantly higher than the historical prevalence of MC in the general population (0.5%)[28]. An intriguing data was that LC and paucicellular LC, considered together, were diagnosed in much more cases than CC (22 *vs* 2, respectively). Usually, literature considered incidence and prevalence of CC higher than LC; however, more recent studies, according to our data, report that the incidence of LC is significantly rising[29].

Patients who underwent colonoscopy were significantly older than those who did not undergo colonoscopy. This could be partially explained considering the age as a parameter associated to augmented risk of malignancy. Hence, clinicians recurred to endoscopy in case of unexplained symptoms and increasing age. However, there was no difference in the median age between patients who did not undergo colonoscopy *vs* those who underwent colonoscopy with multiple biopsies.

Considering biochemical results and symptoms among various groups, only chronic diarrhea was significantly higher in patients with MC than in those who did not undergo colonoscopy (P = 0.03). Thus, in patients with mild duodenal damage only this symptom could predict MC. However, due to its multifactorial pathogenesis, the presence of diarrhea cannot be the only element to decide whether this type of patients should undergo colonoscopy with multiple biopsies. On the other hand, the absence of diarrhea cannot exclude the indication for colonoscopy with multiple biopsies, because only 5 out of 48 (10.4%) patients who had a colonoscopy with biopsies had normal microscopic findings, despite suffering also from abdominal pain, weight loss, constipation, positive fecal occult blood. The search for HLA-DQ2/DQ8 haplotypes seems to be useful, although the data in our retrospective study are not broad enough to provide definitive conclusions. Since this test has a very high negative predictive value in the diagnosis of CD, in patients with mild duodenal damage, negative serological tests for CD and the above reported symptoms, the negativity of HLA-DQ2/DQ8 haplotypes can definitively exclude this disease and propel to search for other etiologies, as MC.

The median age at which the diagnosis of MC was made in our patients (40 years) is lower than literature reports. Such finding may contrast with the idea of MC as disease of the elderly pointing out to a possible underestimation of this condition.

Another element that emerged from this study was the low prevalence of *H. pylori*-infection both in patients with MC (24%) than without MC (34%). The literature reports that *H. pylori* infection is related to duodenal lymphocytosis[9], which disappears after bacterial eradication. At the same time, we have recently found an inverse association between MC and *H. pylori* infection[29]. The results of the present study agree with the fact that in case of mild duodenal damage and MC the prevalence of *H. pylori* infection is lower than the general population (in our case 24% *vs* 47%)[30]. Moreover, the rate of *H. pylori* eradication in this context, is similar to that obtained in the general population[31].

In our study, the role of pharmacological therapy in the pathogenesis of MC is not fully clear. In fact, only 4 patients used PPIs and 1 patient used NSAIDs before the diagnosis of MC. This differs from the well-known data reporting that this type of medications are often implicated as a cause of MC[32], and could be explained by a β error (*i.e.,* the failure to detect an effect that is present) due to the small sample size. Considering the outcome of therapy used to treat MC, budesonide emerged as the best treatment, due to a clinical improvement, in more than 90% of patients. Mesalamine seemed to be a valid therapeutic approach for less severe cases. According to some reports, our results confirm the appropriateness of this management[32].

Although in literature an association between MC and malabsorption is not reported[33], in our study 12 (48%) patients presented signs of it. A potential disease of the small intestine, beyond the duodenum, could explain these features. More efforts are thus needed to understand this clinical condition.

This retrospective analysis shows inadequate habits of clinicians to search for a coproculture or a parasitological test; also the search for *Giardia Lamblia* was out of routine. Such investigations should play an important role in the attempt to identify the cause of duodenal damage. In fact, literature reports that the search for *Giardia Lamblia* or other pathogens should be included in the diagnostic work up of type Ⅰ Marsh-Oberhuber duodenal damage[34].

A potential limitation of our study is its retrospective design with a theoretical loss of balance on parameters analyzed. Nevertheless, we noticed uniform diagnostic and follow-up criteria.

In conclusion, MC is frequently associated with mild duodenal damage. This association may suggest the existence of a "microscopic enterocolitis", and specifically of a "lymphocytic enterocolitis", that involves the entire gastrointestinal tract. It is advisable to perform a colonoscopy with biopsies in all patients with type Ⅰ Marsh-Oberhuber duodenal damage and symptoms as chronic diarrhea, abdominal or epigastric pain, loss of weight, after exclusion of standard causes.

COMMENTS

Background

The diagnosis of celiac disease (CD) is based on the finding of positive antibody tests (anti-endomysium and anti-tissue transglutaminase), confirmed by biopsies that reveal the characteristic duodenal damage. The Marsh-Oberhuber classification is usually used to grade the severity of duodenal lesions, with the type Ⅲ representative of CD. Nevertheless, there are patients with suggestive symptoms of CD, mild duodenal damage [*i.e.,* an increase of intraepithelial lymphocytes (IEL)] defined type Ⅰ, according to Marsh-Oberhuber classification,

and negative antibody tests. This clinical condition, that does not conform with the diagnosis of CD, needs to be investigated for other causes as well as for comorbidities. Microscopic colitis (MC), previously considered rare, was demonstrated as a relatively common cause of chronic, watery, diarrhoea. While some isolated studies reported some association between MC and Marsh I duodenal damage, the interpretation of this finding is poorly described.

Research frontiers

To date, scarce information is available on the association between mild duodenal damage and MC.

Innovations and breakthroughs

This study is the first showing that type I Marsh-Oberhuber duodenal damage is strongly associated with MC, mainly lymphocytic colitis (LC). More than half (52.1%) of the patients who underwent colonoscopy with multiple biopsies had MC. This percentage is significantly higher than prevalence of MC in the general population (0.5%). This association supports the hypothesis of a link between these two entities.

Applications

These findings, of association between type I Marsh-Oberhuber duodenal damage and MC, may suggest the existence of a "microscopic enterocolitis", and specifically of a "lymphocytic enterocolitis", that involves the entire gastrointestinal tract. It is advisable to perform a colonoscopy with biopsies in all patients with type I Marsh-Oberhuber duodenal damage and symptoms as chronic diarrhea, abdominal or epigastric pain, loss of weight, after exclusion of standard causes.

Terminology

The Marsh-Oberhuber classification is usually used to grade the severity of duodenal lesions, with the type III representative of CD. There are patients with suggestive symptoms of CD, mild duodenal damage (*i.e.*, an increase of IEL) defined type I, according to Marsh-Oberhuber classification, and negative antibody tests, that do not conform with the diagnosis of CD. MC is a chronic inflammatory bowel disease, distinct in LC and collagenous colitis. The histological feature of LC is the presence of more than 20 IEL/100 surface epithelial cells (< 5 IEL/100 in the normal colon). Paucicellular LC is a term used when the number of IEL is comprised between 5 IEL/100 and 20/100 surface epithelial cells. In MC, IEL are T-Lymphocyte $CD3^+$ and $CD8^+$, similar to those described in case of type I Marsh-Oberhuber lesions. Here we report for the first time the association between type I Marsh-Oberhuber duodenal damage and MC, arguing for the existence of a possible "microscopic enterocolitis".

Peer-review

This is an interesting manuscript.

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META-ANALYSIS

Hepatitis C infection and renal cell carcinoma: A systematic review and meta-analysis

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Abstract

AIM

To investigate the association between hepatitis C virus (HCV) infection and risk of renal cell carcinoma (RCC).

METHODS

A literature search was performed from inception until February 2016. Studies that reported relative risks, odd ratios, hazard ratios or standardized incidence ratio comparing the risk of RCC among HCV-infected participants *vs* those without HCV infection were included. Participants without HCV infection were used as comparators. Pooled odds ratios and 95%CI were calculated using a random-effect, generic inverse variance method.

RESULTS

Seven observational studies were with 196826 patients were included in the analysis to assess the risk of RCC in patients with HCV. A significantly increased risk of RCC among participants with HCV infection was found with a pooled RR of 1.86 (95%CI: 1.11-3.11). The association between RCC and HCV was marginally insignificant after a sensitivity analysis limited only to studies with adjusted analysis, with a pooled RR of 1.50 (95%CI: 0.93-2.42).

CONCLUSION

Our study demonstrated a potential association between HCV infection and RCC. Further studies of RCC

surveillance in patients with HCV are required.

Key words: Hepatitis C virus; Renal cancer; Kidney cancer; Systematic review; Meta-analysis

Core tip: Hepatitis C virus (HCV) is a leading cause of cirrhosis in the United States with a steadily increasing prevalence over the past two decades. Interestingly, HCV infection may also be associated with an increased risk of renal cell carcinoma (RCC) as observed in several epidemiologic studies. To further investigate this possible association, we conducted this systematic review and meta-analysis of observational studies reporting the risk of RCC among HCV-infected patients. We found a significantly increased risk of RCC among participants with HCV infection with the pooled risk ratio of 1.86 (95%CI: 1.11-3.11).

Wijarnpreecha K, Nissaisorakarn P, Sornprom S, Thongprayoon C, Thamcharoen N, Maneenil K, Podboy AJ, Cheungpasitporn W. Hepatitis C infection and renal cell carcinoma: A systematic review and meta-analysis. *World J Gastrointest Pathophysiol* 2016; 7(4): 314-319 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i4/314.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i4.314

INTRODUCTION

Hepatitis C virus (HCV) remains the most common cause of chronic liver disease and cirrhosis worldwide and is one of the leading causes of chronic hepatitis and cirrhosis in the United States with a steadily increasing prevalence over the past two decades[1,2]. While commonly associated with hepatocellular carcinoma[2], hepatitis C infection is associated with extrahepatic malignancies including cholangiocarcinoma, non-Hodgkin's lymphoma and possibly myeloma[3-5]. The oncogenic properties of hepatitis C are hypothesized to be secondary to chronic antigenic stimulation of the immune system, promotion of a chronic inflammatory state or direct oxidative stress[6]. Besides, chronic HCV infection has also been linked to a myriad of extrahepatic diseases including increasing the risk of renal disease and increasing the prevalence of chronic kidney disease up to 40% higher compared to non-infected patients[7,8].

Renal cell carcinoma (RCC), arising from the renal cortex is responsible for 80% of all renal malignancies and accounts for approximately 14000 deaths each year in the United States[9]. The risk factors for RCC such as acquired cystic kidney disease, smoking, kidney stones, obesity, hereditary factors have been described[10-14]. The epidemiological studies have demonstrated an increasing incidence of RCC, particularly in African Americans[15].

Secondary to the oncogenic nature of Hepatitis C, several studies have linked chronic infection with an increased risk for development of RCC[16-22]. However, the findings from these studies were contradictory. Thus, we performed this meta-analysis to examine the risk of RCC in HCV-infected patients.

MATERIALS AND METHODS

Literature search

Two investigators (Karn Wijarnpreecha and Wisit Cheungpasitporn) independently reviewed published studies indexed in MEDLINE, EMBASE, and the Cochrane database from their inception to February 2016 using the search strategy that included the terms for "hepatitis" and "renal cancer" as described in Item S1 in online supplementary data. No limitation on language was applied. A manual search for additional studies using references of selected retrieved articles was also performed. Three investigators (Karn Wijarnpreecha, Charat Thongprayoon and Wisit Cheungpasitporn) independently reviewed the titles and abstracts of the studies identified in the search based on inclusion and exclusion criteria. The full text of the included studies from the first phase was reviewed independently to ascertain whether or not they matched the inclusion criteria. We also performed a manual search of conference proceedings from major gastroenterology and hepatology meetings for additional abstracts on the topic. When additional information was needed, we contacted the corresponding investigators of eligible studies.

Study selection

The inclusion criteria were as follows: (1) observational studies assessing the association between hepatitis C and RCC; (2) odds ratios, relative risks or hazard ratios with 95%CI were provided; and (3) individuals without HCV infection were used as comparators in cohort studies while individuals without RCC were applied as comparators in the cross-sectional and case-control studies.

Study acceptability was individually defined by the three investigators mentioned above. Disagreements in the ascertainment of study eligibility were settled by joint agreement. Also, the quality of each study was individually appraised by each investigator. We used the validated Newcastle-Ottawa quality assessment scale for cohort and case-control studies[23] and modified Newcastle-Ottawa scale[24] for the cross-sectional study.

Data extraction

A data collection report was utilized to derive the information from each study including name of title and the first author, year of study and publication, country, demographic data of the participants, number of participants, method used to diagnose the HCV infection and RCC, effect estimates (odds ratios, relative risks or hazard ratios) with 95%CI, and factors adjusted in the multivariate analysis. To assure the certainty, this data

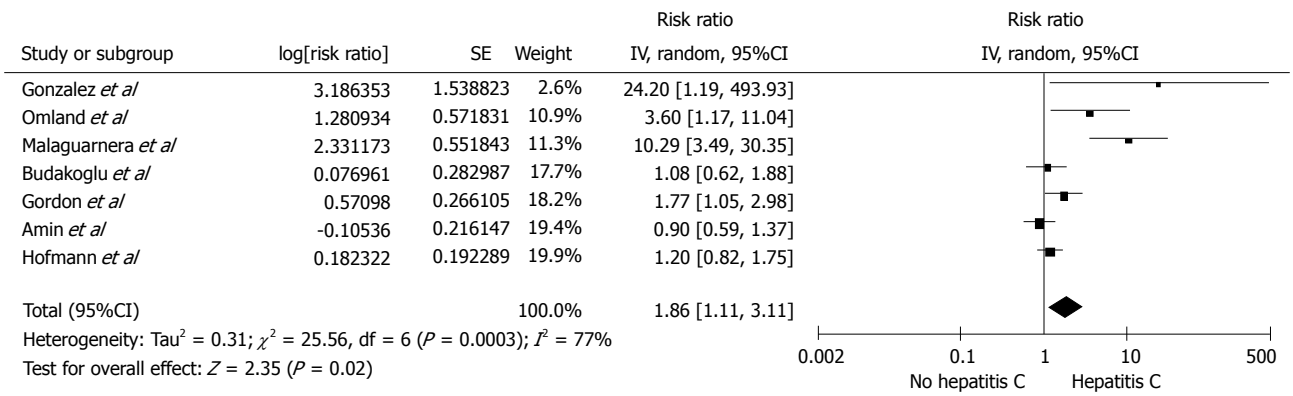

Figure 1 Forest plot of all included studies of the association between hepatitis C infection and renal cell carcinoma. Square data markers represent risk ratios (RRs); horizontal lines, the 95%CI with marker size reflecting the statistical weight of the study using random-effects meta-analysis. A diamond data marker represents the overall RR and 95%CI for the outcome of interest.

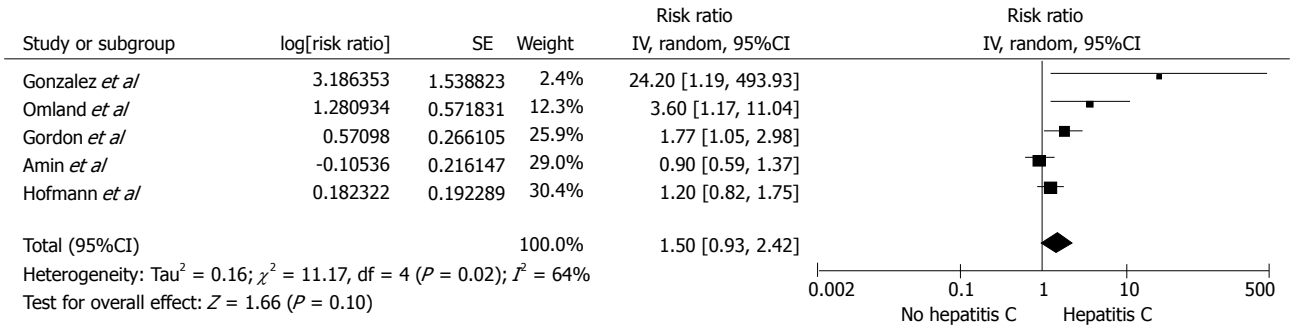

Figure 2 Forrest plot of all included studies in sensitivity analysis of the association between hepatitis C infection and renal cell carcinoma. Square data markers represent risk ratios (RRs); horizontal lines, the 95%CIs with marker size reflecting the statistical weight of the study using random-effects meta-analysis. A diamond data marker represents the overall RR and 95%CI for the outcome of interest.

extraction process was reviewed by all investigators.

Statistical analysis

Review Manager (RevMan) 5.3 software from the Cochrane Collaboration was utilized for meta-analysis. Generic inverse variance (DerSimonian and Laird) method[25] was employed to combined adjusted point estimates and standard errors from each study. We used a random-effect model due to the high likelihood of between-study variance from different study designs and populations. Cochran's *Q* test and I^2 statistic were used to determine the between-study heterogeneity. A value of I^2 of 0%-25%, 25%-50%, 50%-75%, and greater than 75% embodied insignificant, low, moderate and high heterogeneity, respectively[26].

RESULTS

Of 5778 potentially relevant articles, 5582 articles were excluded by the title and abstract not fulfilling inclusion criteria due to the type of article, study design, population, or outcome of interest. Additionally, 189 articles were excluded (35 articles were not observational studies, and 154 articles did not describe the outcomes of interest, Macleod *et al*[27]'s study did not contain data on specific viral hepatitis{Macleod, 2013 #83}). Finally, seven observational studies (4 cohort and 3 case-controlled studies) with 196826 patients were included in the meta-analysis[16-22]. Item S2 describes the study selection flow. The characteristics and quality appraisal of the included studies of HCV and RCC are shown in Table 1. Four studies were conducted in Europe, 2 in the United States, and 1 in Australia.

The risk of renal cell carcinoma in patients with hepatitis C virus infection

Among individuals with HCV infection, there was a significantly increased risk of RCC with the pooled risk ratio (RR) of 1.86 (95%CI: 1.11-3.11, I^2 =77%), as demonstrated in Figure 1. The statistical heterogeneity was high with an I^2 of 77%. The association between RCC and HCV was marginally insignificant after the sensitivity analysis including only studies with confounder adjustment[16,18-20,22] with a pooled RR of 1.50 (95%CI: 0.93-2.42, I^2 =64%), as shown in Figure 2.

Evaluation for publication bias

Two authors (Karn Wijarnpreecha and Wisit Cheungpasitporn) independently performed the assessment of the risk of bias of the included studies. Only minor disagreements between 2 reviewers were present and were resolved by discussion and consensus. A funnel plot was constructed to assess publication bias for the risk of RCC in HCV-infected patients (Figure S1). The

Table 1 Main characteristics of the studies included in this meta-analysis

Ref.	Country	Study design	Year	Total number	Study sample	Exposure definition	Exposure measurement	Outcome definition	Outcome ascertainment	Adjusted OR	Confounder adjustment	Quality assessment (Newcastle-Ottawa scale)
Amin *et al*[16]	Australia	Cohort study	2006	75834 in HCV patients	People notified with HCV infection to the New South Wales Health Department's Notifiable Diseases Database between 1 January 1990 and 31 December 2002 (HCV group) and NSW population (control group)	HCV infection	Detection of anti-HCV antibody or HCV RNA	Renal cancer or kidney cancer	The NSW Central Cancer Registry with ICD-10 code C64 for kidney cancer and C65 for renal cancer	Kidney cancer 0.9 (0.6–1.4)	Age, sex and calendar year	Selection: 4 Comparability: 1 Outcome: 3
Malagu arnera *et al*[21]	Italy	Case control study	2006	315 (15 case and 300 control)	Elderly kidney cancer patients attending geriatric department (case) and elderly volunteers (control)	Positive Anti-HCV	Detection of Anti-HCV antibody using enzyme-linked immunosorbent assay. Assay positive samples were confirmed by immunoblotting	Kidney cancer	N/A	10.29 (3.49-30.36)	None	Selection: 3 Comparability: 0 Outcome: 2
Omland *et al*[22]	Denmark	Cohort study	2010	4204 in HCV patients	Acute or chronic HCV infected patients listed in the Danish National Hospital Registry between 1994 and 2003 without previous diagnosis of cancer (HCV) and general population (control)	Acute or chronic HCV infection	ICD-10 code (B17.1 and B18.2) from the Danish National Hospital Registry	Kidney cancer	The Danish Cancer Registry with ICD-7 code 180	3.60 (0.98–9.22)	Age, sex and year of diagnosis	Selection: 4 Comparability: 1 Outcome: 2
Gordon *et al*[19]	United States	Cohort study	2010	67063	HCV-tested patients (both positive and negative) between 1997 and 2006 from administrative data from Henry Ford hospital	HCV infection	Positive anti-HCV test using enzyme-linked immunosorbent assay, confirmed by a documented positive molecular assay for HCV RNA	Renal cell carcinoma	Health system cancer registry, confirmed by medical record and pathology report review	1.77 (1.05-2.98)	Age, race, gender, chronic kidney disease	Selection: 4 Comparability: 1 Outcome: 3
Budakoğlu *et al*[17]	Turkey	Case-control study	2011	6170 (903 case and 5267 control)	Patients who had histologically proven renal cell carcinoma diagnosis between 2005 to 2010 from six tertiary cancer centers (case) and healthy people who were living in the same geographic regions (control)	Positive anti-HCV	Positive anti-HCV test using enzyme-linked immunosorbent assay	Renal cell carcinoma	Histopathology report	1.08 (0.62-1.88)	None	Selection: 3 Comparability: 0 Outcome: 3
Hofmann *et al*[20]	Sweden	Cohort study	2011	43000 in HCV patients	All Swedish residents diagnosed with HCV infection between 1990 and 2006 (HCV group) and general population (control)	Chronic HCV infection	The national surveillance database at the Swedish Institute for Infectious Disease Control	Kidney cancer	The national Cancer register with ICD-7 code 180.0 and 180.9 and histologic confirmation	1.2 (0.8–1.7)	Age, sex and calendar year	Selection: 4 Comparability: 1 Outcome: 3
Gonzalez *et al*[18]	United States	Case-control study	2015	240 (140 case and 100 control)	Newly diagnosed renal cell carcinoma (case) and newly diagnosed colon cancer patients (control)	Chronic HCV infection	Detection of anti-HCV antibody or HCV RNA	Renal cell carcinoma	Histopathology report	Positive HCV RNA 24.20 (2.4 - > 999.9)	Sex, age, race, BMI, smoking, alcohol abuse, hypertension, diabetes mellitus, dyslipidemia, coronary artery disease, congestive heart failure, chronic kidney disease, cirrhosis	Selection: 3 Comparability: 2 Outcome: 3

BMI: Body mass index; DNHR: Danish National Hospital Registry; ELISA: Enzyme-linked immunosorbent assay; HCV: Hepatitis C virus; N/A: Not available; NSW: New South Wales; RCC: Renal cell carcinoma; SIR: Standardized incidence ratio.

funnel plot was suggestive of a small publication bias toward studies with a positive correlation between HCV infection and RCC.

DISCUSSION

This meta-analysis was conducted to summarize all presently available data on the association between HCV infection and RCC. Our study demonstrated a 1.86-fold increased risk of RCC among participants who had HCV infection compared to those without HCV infection. Our analysis also illustrates that that RCC patients with HCV were significantly younger than RCC patients without HCV. The results of our study reinforce the hypothesis that HCV may accelerate the risk of developing RCC.

Although the nature in which HCV induces RCC is not entirely understood, several hypotheses exist. A recent bioinformatics study demonstrated a plausible biological relationship between HCV infection and the development of RCC *via* the NY-REN-54 protein. The NY-REN-54 protein which is an altered ubiquitin-related protein that plays a role in the disturbance impairs the autophagic response *via* to the ubiquitin-protein ligase-related self-regulatory mechanism, which in turn promotes oncogenesis[28]. Additionally, inhibition of cytotoxic T-lymphocyte-dependent apoptosis by the hepatitis virus secondarily leads to a disturbance in host immunity and normal tissue homeostasis leading to carcinoma formation[29]. Lukkonen *et al*[30] demonstrated an increased expression of serine protease inhibitor Kazal (SPIK), a cellular protein that inhibits serine protease-related apoptosis, in RCC tissue samples as an additional mechanism for HCV-induced RCC.

There are several limitations in our study. Firstly, there was a high amount of statistical heterogeneity present in the completed analysis. The potential source of this heterogeneity includes variation in confounder-adjusted methods (*e.g.,* age, sex, ethnicity, and chronic kidney disease), exposure measurement, outcome ascertainments, and follow-up duration. Secondly, it should be noted that there was a potential small publication bias with a positive association between HCV infection and RCC. The possibility of selection bias could play a role in chart-reviewed population base study. Finally, this meta-analysis of observational studies could only show an association. It cannot establish causality as an unknown number of confounders could play a role in the association between HCV and RCC.

In summary, this study demonstrates a potential association between HCV infection and RCC. In the new era of treatment of HCV infection, direct-acting antiviral agents have been demonstrated to be effective therapy and increasingly used[31]. These agents may potentially decrease the incidence of RCC in HCV patients in the long term.

COMMENTS

Background

Hepatitis C virus (HCV) is one of the leading causes of cirrhosis as the prevalence of HCV has been steadily increasing over the past two decades in the United States. Extrahepatic manifestations of HCV are common. Interestingly, HCV infection could also increase the risk of renal cell carcinoma (RCC) as observed in several epidemiologic studies.

Research frontiers

The results of those epidemiologic studies were inconsistent. To further investigate this possible association of HCV and RCC, the authors conducted this systematic review and meta-analysis of observational studies reporting the risk of RCC among HCV-infected patients.

Innovations and breakthroughs

The authors found a significantly increased risk of RCC among participants with HCV infection with the pooled risk ratio (RR) of 1.86 (95%CI: 1.11-3.11). The sensitivity analysis including only studies with confounder adjustment also demonstrated increased risk of RCC among participants with HCV infection with the pooled RR of 1.50 (95%CI: 0.93-2.42) even though without reaching statistical significance.

Applications

This study demonstrated a potential association between HCV infection and RCC. This finding suggests that a history of HCV is potentially associated with RCC and may impact clinical management and cancer surveillance.

Peer-review

The manuscript is an interesting meta-analysis about the correlation of HCV infection and RCC development. The aim of the meta-analysis is clearly stated and methods are well-described.

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