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Small Intestinal Bacterial Overgrowth in Gastric Cancer Patients in Relation to *Helicobacter pylori* Status and Gastric Cytokine Levels

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ABSTRACT

Objective: As Small intestinal bacterial overgrowth (SIBO) effects treatment decisions and quality of life of patients, information on the prevalence of SIBO in gastric cancer patients pertaining to predicting the development of SIBO from *H. pylori* status and effects of SIBO on gastric immunity is urgently needed. In this study, we attempt to estimate the prevalence of SIBO and examine the relationship between these factors in gastric cancer patients.

Methods: Eighty gastric cancer patients and thirty healthy controls were included in this prospective study. Preoperative glucose hydrogen breath tests were performed to diagnose SIBO and a urea breath test was performed to establish *H. pylori* status. Interleukine-6 (IL-6) and tumor necrosis factor-α (TNF-α) expression in tumor tissues was assessed by immunohistochemistry (IHC) in gastrectomy specimens. The correlation between SIBO and outcome measures was investigated.

Results: Gastric cancer is significantly associated with SIBO (45% *vs.* 6.67%, P<0.05), while hydrogen-breath-test-positivity was associated with Helicobacter-positivity (r=0.397, P<0.05). IL-6 and TNF-α expression were significantly higher in SIBO-positive patients than in SIBO-negative patients (P<0.05). Three-year cumulative survival rates for SIBO-positive patients were significantly lower than for SIBO-negative patients (P<0.05).

Conclusion: This study indicates that SIBO is common among gastric cancer patients, and is associated with poor outcome. *H. pylori* infection may play an important role in the development of SIBO. SIBO in poor performance patients with gastric cancer potentially relates to high gastric IL-6 and TNF-α levels in this patient group.

INTRODUCTION

Gastric adenocarcinoma is the fourth most common malignant carcinoma and the second leading cause of cancer-related death in the world [1,2]. A clear relationship between *Helicobacter pylori* (*H. pylori*) infection and the development of gastric cancer has been established [3]. Whether gastric or intestinal overgrowth with other bacteria can influence stomach carcinogenesis, however, remains unknown. Generally speaking, SIBO is expected in post-gastrectomy patients, and it has been indeed reported that SIBO is common among these patients [4,5]. However, it remains unclear whether SIBO in this patient group was pre-existing or is a consequence of surgical intervention, and how the presence of SIBO affects disease outcome. Further insights into the relationship between SIBO and gastric cancer are urgently needed, in view of the established importance of bacterial infection in general and the therapeutic consequences of the presence of SIBO.

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A potential causative role of SIBO comes from the associated immunological responses. Inflammation, as many studies have indicated, plays a vital role in promoting tumor progression [6]. In this aspect, both TNF-α and IL-6 are interesting, as they have been linked to tumor progression in the gastrointestinal system [7]. For instance, IL-6 can induce vascular endothelial growth factor synthesis to promote angiogenesis, and foster tumor cell adhesion and invasion to promote tumor growth and metastasis [8]. Both cytokines are produced in response to bacterial cell wall endotoxins, and thus, clearly relate to SIBO [9]. Accordingly, in NASH patients, SIBO-derived increase in portal endotoxins has been directly linked to the production of these inflammatory cytokines [10]. However, how SIBO in gastric cancer patients relates to cytokine levels remains unexplored.

Interest in the potential detrimental role of SIBO in gastrointestinal cancers has grown due to the myriad of recent studies, in which detrimental changes in fecal microbiota composition have been linked to both the increase in proinflammatory cytokine levels and development of cancer *per se* [11]. In particular, a recent study carried out by Biagi et al. provided evidence that dysbiosis, which involve the excess proliferation of Proteobacteria and concomitant reduction in Firmicutes and Bacteroidetes, causes IL-6 and IL-8 levels to increase, linking microbiota to cancer [12]. Thus, it is fair to say that substantial momentum with respect to aberrant bacterial growth, subsequent changes in local cytokine levels, and the development of gastrointestinal malignancies exists. This further prompts studies to investigate the relationship between SIBO and cytokine levels with respect to gastric cancer.

The above-mentioned considerations encouraged us to initiate this study on the prevalence of SIBO in gastric cancer patients, and its relationship to cytokine levels and *H. pylori* status. Our results reveal that SIBO is common in pre-operative gastric cancer patients, which relates clinically to poor prognosis and mechanistically to *H. pylori* status and increased cytokine levels. Thus, our results reveal the unexpected importance of SIBO in gastric cancer.

MATERIALS AND METHODS

Patients

Eighty patients with histopathologically confirmed gastric cancer that presented at our department from January 2012 to January 2015 were enrolled in this study. This cohort consisted of 67 male and 13 female patients (age range, 30-82 years), and patients were scheduled to receive gastrectomy. Thirty healthy volunteers were also analyzed as controls. This study was conducted at Qingdao Municipal Hospital and was approved by the Institutional Research Ethics Board of Qingdao Municipal Hospital. Informed consent was obtained from all patients. Characteristics of our cohort are shown in **Table 1**. Exclusion criteria for this study were as follows: history of chemotherapeutic therapy, history of diabetes, presence of thyroid disease, or previous gastrointestinal surgery; patients taking anti-secretory agents such as proton pump inhibitors or histamine H_2 receptor antagonists, antibiotics, probiotics and prokinetics; patients with renal insufficiency, major psychiatric disease, hearing impairment, or masticatory dysfunction; and patients who underwent colonoscopy within the past three months.

Table 1. Clinical and demographic characteristics of the study cohort.

Number of patients	80
Age (years)	
Range	43-83
Median	65.3
Gender (%)	
Male	67 (83.8)
Female	13 (16.2)
Nodal status, *n* (%)	
N_0	34 (42.5)
N_1	46 (57.5)
Depth of invasion, *n* (%)	
T_1	47 (58.7)
T_2	33 (41.3)
Grading, *n* (%)	
1	44 (55)
2-3	36 (45)

Glucose hydrogen breathe test for detecting SIBO: Testing for SIBO was always made prior to gastrectomy. Patients were instructed to eat a low fiber diet for three days before commencing the glucose hydrogen breath test. Subsequently, 50 g of glucose in 200 ml of water was administered after taking a basal-end expiratory breath. Then, breath samples were collected at 20-minute intervals for two hours. An increase in breath H_2 concentration ≥ 12 ppm over baseline value in two consecutive readings within two hours was defined as SIBO.

Cytokine Assays

IL-6 and TNF-α expression in tumor tissues obtained during the operation were measured by immunohistochemistry (IHC). Immunohistochemically stained SIBO-positive and SIBO-negative samples from patients were simultaneously processed to allow

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a proper comparison. Both rabbit related IL-6 (diluted at 1:200) and TNF-α (diluted at 1:200) antibodies were obtained from Bioss (Beijing; China). Microscopic evaluation of stains in both cytokines was independently performed by two pathologists. Each tumor was given a score, which included both staining intensity (no stain=0, low level immunoreactivity=1, moderate immunoreativity staining=2, and strong staining=3) and the percentage of stained cells (0%=0, <10%=1, 10-50%=2, 51-80%=3, and >80%=4), and the final number was reached through multiplication of the parameters. For statistical analysis, the 12-tier scoring was simplified by collapsing results on three categories (0-4, weak positive; 5 and 6, moderate positive; 8-12, strong positive [13]). We also studied the association between IL-6 and TNF-α expression and outcome.

C13-UBT for *H. pylori*

H. pylori status of each patient was determined using C13-UBT due to its obvious advantages such as its noninvasive nature and its easiness to perform; and because it the gold standard for determining the *H. pylori* status of a patient. Sensitivity and specificity of the test were 94% and 83%; respectively the first initial breath sample was collected after fasting for at least eight hours. Then, a 100 mg tablet of 13 C-urea (free of citric acid) was orally administered. Breath samples were collected in the sitting position using special breath collection bags before 13C-urea administration (baseline) and 20 minutes after administration. The collected breath samples were analyzed using an isotope-selective non dispersive infrared spectrometer. Results over the 4.4 delta over baseline (DOB) were considered positive for *H. pylori* infection.

Statistical Analysis

The X2-test was used to statistically analyze cytokine levels between SIBO-positive and SIBO-negative gastric cancer patients. It was also used to analyze the presence of SIBO between gastric cancer patients and healthy controls, and establish the correlation of SIBO with IL-6, TNF-α and *H. pylori*. Survival curves of patients were plotted using the Kaplan-Meier method, and differences were assessed by log-rank test. All statistical analyses were performed by SPSS version 17.0 for Windows groups (SPSS Inc., Chicago, IL, USA). The difference was considered significant at $P<0.05$ level.

RESULTS

Incidence of SIBO in Patients with Gastric Cancer and Controls

Among all gastric cancer patients, 36 patients (45%) were tested positive for SIBO through hydrogen breath test **(Figure 1a)**; and among these SIBO-positive patients, 10 patients (27.8%) were over 60 years and 26 patients (72.2%) were less than 60 years **(Figure 1b)**. In the control cohort, only two patients exhibited SIBO. The incidence of SIBO in tumor patients over 60 years (10 patients, 62.5%) was significantly higher ($P<0.05$), compared to patients less than 60 years old (26 patients, 40.6%; **Figure 1b**). Gastric cancer patients had statistically and significantly more SIBO. Thus, SIBO is common in gastric cancer. Subsequently, we initiated experiments to address the functional consequences of SIBO on gastric immunity.

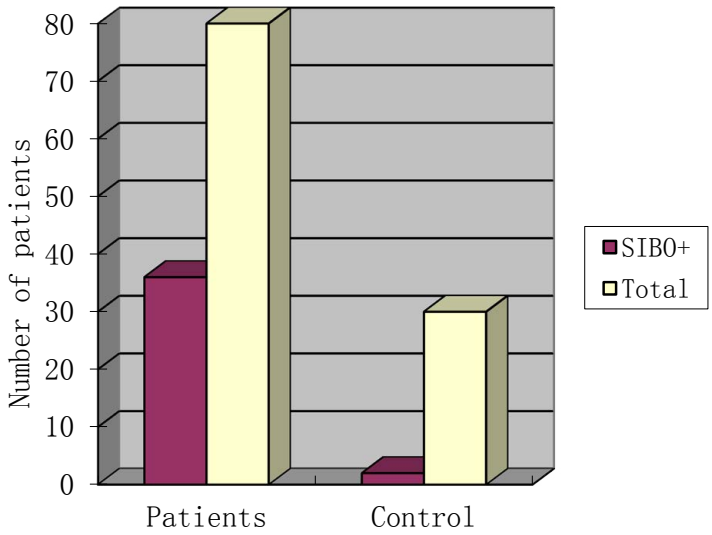

Figure 1A. Presence of SIBO in gastric cancer patients and controls. Patients and controls were analyzed as described in the methods and expressed as number of individuals positive for bacterial overgrowth and total number of patients.

Cytokine Expressions in Gastric Cancer Patients

Resection specimens obtained from our study cohort were investigated for local cytokine levels through IHC. Due to the close link to both gastrointestinal cancer and bacterially driven inflammation, we focused in investigating IL-6 and TNF-α. Examples of staining are shown in **Figures 2A1 and 2B1).** In our cohort, we observed that 48 of 64 (75%) patients <60 years old displayed moderate to strong IL-6 and TNF-α expressions; while 11 (68.8%) and nine (56.3%) patients >60 years old had such IL-6 and TNF-α scores, respectively **(Table 2).** Thus, there was no significant difference in IL-6 and TNF-α expression with age. Similarly,

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there was no significant difference in gender, but the expression of these cytokines is the characteristic of gastric cancer in our cohort.

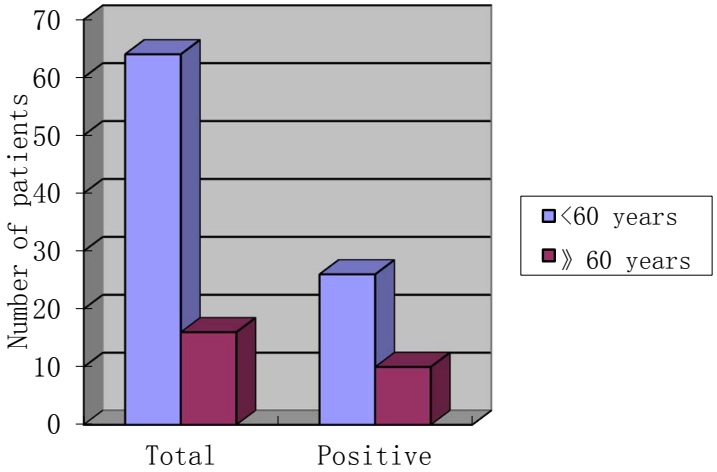
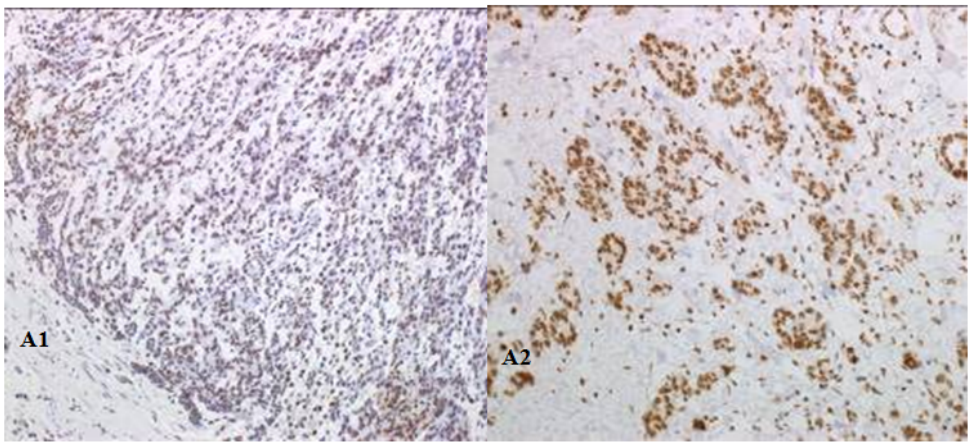
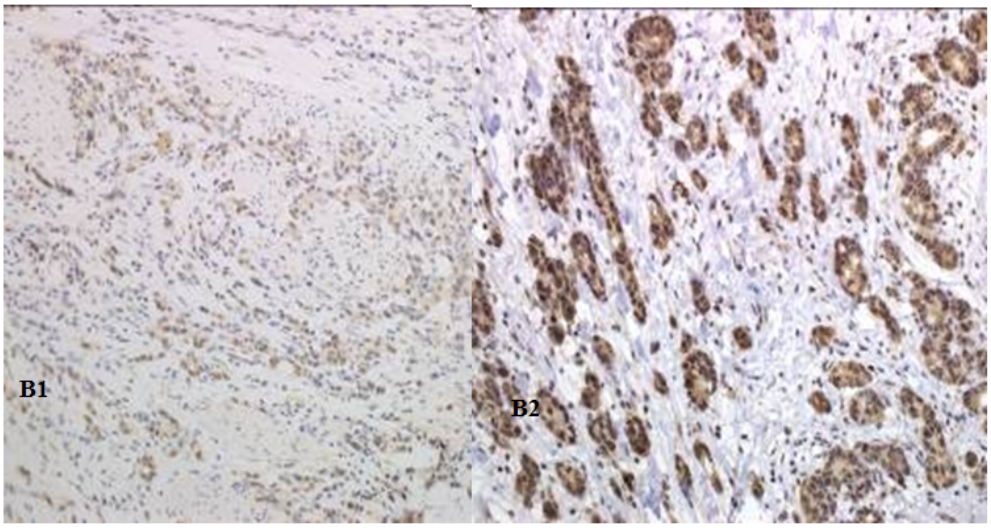


Figure 1B. Positivity for SIBO in our study cohort as assayed using the hydrogen breath test. Results were stratified by age.



IL-6 immuonoreactivity



TNF-α immunoreactivity

Figure 2. Examples of microscopic findings are shown. (A1) IL-6 expression in the SIBO-negative group; (A2) IL-6 expression in the SIBO-positive group; (B1) TNF-α expression in the SIBO-negative group; (B2) TNF-α expression in the SIBO-positive group; (A) x200; (B) x200.

The number of patients, in which an IL-6 cytokine response was evident, was significantly higher (P=0.002) in N_1 patients (40), when compared to N_0 patients (19). Thus, it appears that a more severe disease is associated with a stronger inflammatory component. In apparent agreement, a similar relation was found with respect to TNF-α expression (P=0.02). Similarly, IL-6

e-ISSN:2320-3528
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expressions were different in T_1-staged *versus* T_2-staged patients (P=0.044); as 32 (68.1%) IL6- stained positive patients were staged as T_1 and 27 (81.8%) IL6- stained positive patients were staged as T_2. However, there was no difference with respect to the level of TNF-α expression and depth of invasion (P=0.212). Further, a significant relationship could be seen between patients classified as G_1 or G_{2-3} with regard to TNF-α levels (P=0.021), while no relationship was found between grading and IL-6 expression levels (P=0.459). *In toto*, it is fair to say that a clear relationship exists between the presence of an inflammatory cytokine environment and more advanced diseases. Subsequently, we investigated the relationship between such inflammation and SIBO.

Table 2. IHC results on cytokines in gastric cancer patients

		IL-6 IHC results (IHC score)		P	TNF-α IHC results (IHC score)		P
		Weak	Moderate-Strong		Weak	Moderate-Strong	
	Cases						
Age							
<60 years	64	16	48		16	48	
>60 years	16	5	11	0.611	7	9	0.138
Gender							
Male	67	17	50		20	47	
Female	13	4	9	0.686	3	10	0.621
Nodal status							
N_0	34	15	19		16	18	
N_1	46	6	40	0.002	7	39	0.02
Depth of invasion							
T_1	47	15	32		16	31	
T_2	33	6	27	0.044	7	26	0.212
Grading							
G_1	44	13	31		8	36	
G_{2-3}	36	8	28	0.459	15	21	0.021

Note: 0-4score: weak; 5 and 6 score, moderately positive; 8-12: strong positive

Cytokine Levels in SIBO-Positive and SIBO-Negative Subjects

Among the 80 gastric cancer patients, 59 (73.8%) patients revealed a moderate-strong expression of IL-6 and 57 (71.3%) patients revealed a moderate-strong expression of TNF-α; while among the SIBO-positive patients, 31 (86.1%) and 30 (83.3%) patients revealed a moderate-to-strong expression for IL-6 and TNF-α, respectively. Conversely, in the SIBO-negative group, only 28 (63.6%) and 27 (75.0%) patients revealed clear staining for these cytokines (IL-6 and TNF-α, respectively); resulting in a significant difference (P<0.05) in IL-6 and TNF-α expressions between SIBO-positive and SIBO-negative patients **(Table 3 and Figure 3)**. Thus, it appears that the presence of SIBO in gastric cancer patients has important consequences for the nature of peri-tumoral cytokine milieu.

Table 3. Comparison of pro-inflammatory cytokine levels in SIBO-positive and SIBO-negative gastric cancer patients.

	IL-6 IHC results (IHC score)		P	TNF-α IHC results (IHC score)		P
N	Weak	Moderate-Strong		Weak	Moderate-Strong	
$SIBO^+$	5	31	0.023	6	30	0.031
$SIBO^-$	16	28		17	27	

Note: SIBO+, SIBO-positive; SIBO- ,SIBO negative; N, number of patients

Correlation of SIBO with *H. Pylori*

H. pylori is a major risk factor for gastric cancer. However, how *H. pylori* status relates to growth of other bacteria in general and to SIBO in particular remains unexplored. In our gastric cancer cohort, we observed that 48 (60.0%) of 80 patients were *H. pylori* positive; wherein, 30 (62.5%) patients were also SIBO-positive, and only six (18.8%) patients tested positive for SIBO in the *H. pylori* negative group. These results in a positive correlation between SIBO and *H. pylori* in gastric cancer patients (r=0.396, P<0.05; **Table 4)**, and thus, *H. pylori* status is a major risk factor for SIBO development in this patient group.

Outcomes

Analysis of survival was conducted in 80 patients in our cohort. At the time of censoring (three years), 44 patients died. We assessed the correlation between the prevalence of SIBO and outcome (survival time). Three-year cumulative survival rates for patients with SIBO-positive were significantly lower than rates for patients with SIBO-negative, as assessed by log-rank test **(**P<0.05, **Figure 3)**. Thus, SIBO may play an important role on outcome for gastric cancer patients.

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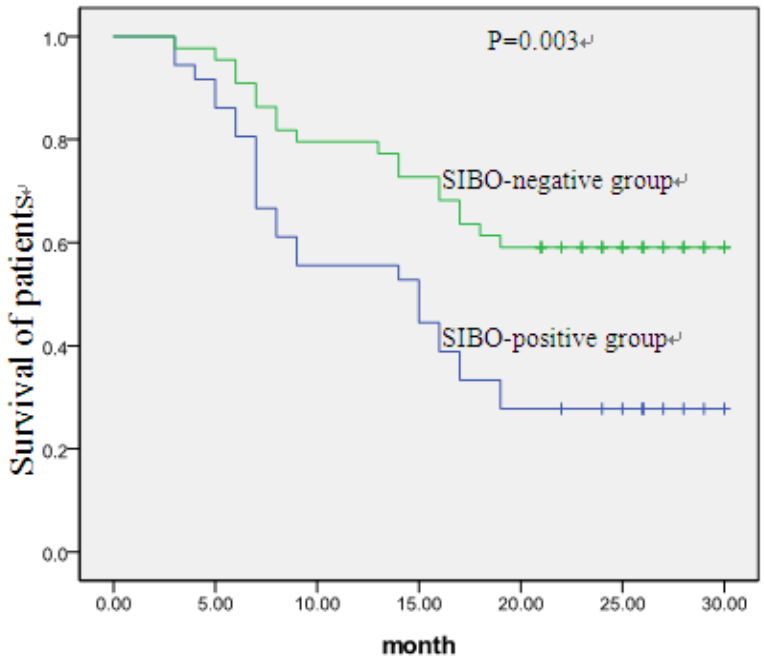

Figure 3. Kaplan-Meier analysis of survival in the study cohort. Results are analyzed with respect to. prevalence of SIBO. The presence or absence of SIBO results in a significant effect on survival for gastric cancer.

Table 4. Correlation of SIBO with *H. pylori.*

	N	$SIBO^+$	$SIBO^-$	r	P
HP^+	48	30	18	0.396	0.001
HP^-	32	6	26		

Note: HP, *H. pylori*; N, number of patients; r, regression coefficient

DISCUSSION

Chronic alteration of intestinal microbiota homeostasis promotes many diseases, including cancer. In particular, the resulting chronic inflammation can profoundly alter local immune responses, causing altered tissue homeostasis, finally leading to cancer. How this general principle relates to SIBO and gastric cancer remains unexplored. In post-gastrectomy patients, high SIBO incidence has already been documented (77.6%), and its relationship with pre-operative SIBO has especially become obscure [4]. Remarkably, in this study, we have demonstrated that SIBO is common in patients with unoperated gastric cancer, which relates too poor outcome. These results call for the pre-operative assessment of SIBO status in gastric cancer patients, and potentially for its treatment upon detection.

Jejunal aspiration is the gold standard for confirming small intestinal bacterial overgrowth [14]. However, invasiveness is the key limitation to jejunal aspiration, as well as contamination from other sources, which remains a problem. In view of these concerns, a simple and effective hydrogen breath testing has become increasingly accepted. Such breath testing can be either a glucose breath test (GBT) or lactulose breath test (LBT), but GBT is the most accurate among the breath testing modalities for the diagnosis of suspected SIBO (diagnostic accuracy of 71.7 for GBT *vs.* 55.1 for LBT) [15]. Therefore, non-invasive GBT was used to evaluate SIBO in gastric cancer patients, and there is no reason to assume that jejunal aspiration would have yielded substantially different results. Thus, we feel that our observations for SIBO in gastric cancer are valid.

There may be various reasons for SIBO in gastric cancer patients such as reduced gastric acid secretion, weakened gastrointestinal motility, damage to the intestinal microenvironment, and impaired local gastrointestinal local immunity; which may all be involved in provoking colonic bacteria to move up to the small intestine and induce SIBO [16]. Sjostedt et al. [17] found that an increased quantity of gut bacteria in gastric cancer patients, probably due to higher pH in the stomach, which may be favorable to bacterial growth. We speculate that the resulting bacterial overgrowth destroys local micro ecology, provoking fulminant inflammatory responses; and in turn, aggravates or even initiates the disease [18]. SIBO is associated with a variety of relatively specific and common gastrointestinal symptoms including bloating, flatulence, diarrhea, abdominal cramping, nausea, and weight loss. Thus, it could negatively affect the patient's well-being and outcome such as by hampering food uptake and subsequent harm to nutritional status. Obviously, more attention should be paid to potential SIBO, and it should probably be treated in gastric cancer patients. In apparent support, Bustillo et al. detected SIBO in a patient with pancreatic cancer. The patient was given 400 mg of norfloxacin every 12 h for five days, and reported the resolution of diarrhea at the 4th day [19]. Thus, we feel that the detection of SIBO status in gastric cancer patients is indicated and requires vigilant monitoring.

H. pylori is known as a class I risk factor (a definite carcinogen) for gastric adenocarcinomas [20,21]. In apparent agreement, this present study also detected a high rate (60%) of *H. pylori* infection among the 80 gastric cancer patients. Furthermore, a positive *H. pylori* status correlated with the presence of SIBO. The reason for this co-presentation remains unclear, but may

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relate to the reduced secretion of gastric acid in *H. pylori* positive patients. The small intestine is relatively sterile under normal circumstances due to the sterilization effects of gastric juice. Thus, *H. pylori*-related increase in pH value can reduce protection from SIBO [22]. Alternatively, *H. pylori* infection can cause gastric mucosal atrophy, intestinal metaplasia and atypical hyperplasia; resulting in damage to the gastrointestinal mucosa, which reduces the antibacterial activity of the stomach [1], leading to symbiotic bacterial overgrowth and the proliferation of harmful bacteria [23]. Finally, parasite and *H. pylori* infections have been shown to shift the composition of intestinal bacteria and increase the diversity of gastric microbiota, which is also potentially predisposing to bacterial overgrowth [24-27]. Determining which of these factors explains the association of SIBO to *H. pylori* infection requires further investigation.

Whatever is the cause of SIBO in gastric cancer patients, the result is probably inflammation; which may promote gastric cancer progress, both with respect to initiation and outcome [8,28,29]. In apparent support, the obtained cytokine data has shown a link between SIBO and IL-6 and TNF-α expression in tumor tissues. Thus, we suggest that further mechanistic studies should be carried out to clarify the relationships demonstrated in this study.

CONCLUSION

In summary, we found an increased prevalence of SIBO in gastric cancer patients compared to control subjects. *H. pylori* apparently predisposes to SIBO in gastric cancer patients. SIBO is associated with TNF-α and IL-6 expressions, and negatively affects the prognosis of gastric cancer. Our results call for both investigations to SIBO status in gastric cancer, and also for its potential treatment.

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All of the authors have contributed in the collection, analysis, and interpretation of data, besides; the authors are agreement in submitting the manuscript for publication.

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