Original Article

Prognostic Role of Intragastric Cytopathology and Microbiota in Surgical Patients with Stomach Cancer

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Abstract

Background: In the last decade, analysis of malignant cells and flora in gastric lavage (GL) has provided interesting data on pathogenesis of gastric cancer (GC). For this study, combining such two aspects into one cyto-microbiologic category, we tested the prognostic role of the presence/absence of cancer cells (GL1/GL0) and bacterial microbiota (MB1/MB0) in our GC population. **Material and Methods:** Between April 2012 and August 2019, 79 surgical patients with GC were prospectively investigated with the determination of GL MB. **Results:** Compared with GL1 MB0, GL1 MB1 strongly correlated with advanced GC, portended poorer overall survival (OS) (45.8 months *vs* 20.5 months, $P = 0.049$), and resulted a significant ($P = 0.008$) and an independent ($P = 0.013$) prognostic factor unfavorable for OS. **Conclusion:** In the light of our results, the cyto-microbiologic parameter of GL MB should be used to gain a better prognosis of GC patients. Administration of antimicrobial treatment for MB1 subjects should be entertained because it could reduce the risk of oncogenesis.

Keywords: Fluid cytology, gastric cancer, gastric microbiota, gastrointestinal cytology, non-gynecologic cytopathology

Introduction

Diverging from other adenocarcinomas affecting the enteral tube, gastric cancer (GC) carcinogenesis is poorly understood impeding the identification of efficient measures for early diagnosis, curative treatment, and reliable prognosis.[1-4] Consequently, as of 2021, GC is still the third leading cause of cancer-related mortality in the world (783.000 deaths per year).[5,6] Since the last decade, cytologic and molecular analysis of gastric lavage (GL) of GC patients has provided interesting results.[7-17] Gastric bacterial microbiota (MB) represents another original issue for GC research drawing medical attention.[18-22] The stomach lumen, in fact, is not sterile and physiologically hosts a rich MB (approximately 10^2–10^4 colony forming units per gram content) mainly composed of the genus *Lactobacillus*, *Clostridium*, *Propionibacterium*, *Streptococcus,* and *Staphylococcus*.[23] In the presence of *Helicobacter pylori* (*H. pylori*)-positive gastritis and pre-cancerous lesions, MB composition is deeply subverted with an important increase of *Lactobacillus*, *Clostridium*, and *Pseudomonas* and a major decrease of *Streptococcus* and *Bacteroides*.[24] Subsequently, penetrating through epithelial mucosa and activating immune system activation, these taxa could co-promote tumor transformation and growth in concert with *H. pylori* and other factors.[18,25] In this study, we combined endoluminal cytology and microbiology into one examination and investigated the clinicopathologic significance and prognostic role of this mixed innovative item: the "GL MB" parameter.

Materials and Methods

We prospectively analyzed the clinicopathologic data of 79 GC patients who were admitted between April 2012 and August 2019 to our Division of General Surgery. Our study followed the principles of the Declaration of Helsinki (as revised in

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Brazil 2013); individual informed consent was obtained from all participants before enrolment. All the participants have been followed until April 2020 or death. All the procedures of nasogastric tube insertion with subsequent GL were conducted by the same operator: in brief, under general anesthesia and before surgical act, the GL was collected under sterile conditions through a nasogastric tube and immediately transported to laboratory and cytopathology service.[15] The following cytomorphological criteria were considered pathognomonic of malignancy: nuclear changes (atypia, anisokaryosis), increased and/or abnormal mitotic figures, high nucleus-to-cytoplasm ratio, nucleolar hypertrophy or multiplicity, highly condensed nuclear chromatin, cytosolic vacuoles (signet-ring cells), pleomorphism, hypertrophy, presence of aggregates, and pseudopapillary [Figure 1].[9] Gastric microbiota (cocci, bacilli, hyphae, and spores) was microscopically evaluated on the same smears prepared for cytologic examination and stained according to the Papanicolaou method [Figures 2 and 3].[26] *Helicobacter pylori* (*H. pylori*) status was further examined in those GL samples showing bacilli by our bacteriology laboratory technicians; bacterial features such as Gram-negative staining, helical or spiral shape, flagellar filaments, diameter of about 0.5 μm, and positive correlation with preoperative gastric biopsies were considered consistent with the microbiologic diagnosis of *H. pylori* infection. Histopathology of surgical specimens was described following the 8th ed.ition of AJCC TNM Staging System.[27] Metastatic lymph node ratio (LNR) was classified into a 4-tier system: LNR0 (0.0), LNR1 (>0–0.3), LNR2 (>0.3–0.6), and LNR3 (>0.6).[9]

Statistics

Statistical analysis was performed using MedCalc Statistical Software version 19.4.1 (MedCalc Software Ltd, Ostend, Belgium). Categorical, ordinal, and continuous variables were compared using the Chi-square, Kruskal-Wallis, logistic regression, Pearson correlation coefficient, and Student's *t*-test. Overall survival (OS) was evaluated as the time from GL collection to death from any cause.[7,9] Survival curves were interpreted and compared through the Kaplan-Meier method and the log-rank test. Univariate and multivariate analyses were performed with one-way ANOVA test and Cox proportional hazards model to identify powerful association and independency among prognostic factors. *P* values <0.05 were considered statistically significant.

Results

The main clinicopathologic characteristics of the studied population as well as the associations with the "GL MB" parameter are listed in Table 1. Considering all the entertained subgroups (GL1 MB1, GL1 MB0, GL0 MB1, and GL0 MB0), the median follow-up was 17.8 months (range: 62–0). Among the 39 patients with GL malignant cells (GL1) (49%), bacterial microbiota was present (MB1) and absent (MB0) in 33 and 6 patients, respectively. In the group without GL cancer cells (GL0) (51%), MB1 was found in 32 and MB0 in 8 cases. At a median follow-up of 33.9

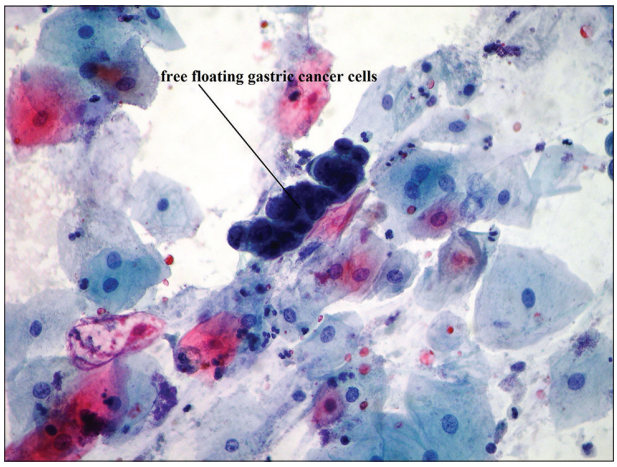

Figure 1: Cluster of gastric cancer cells exfoliated into gastric lavage (Papanicolaou stain, 9100 oil immersion. Magnification: 44× field of view)

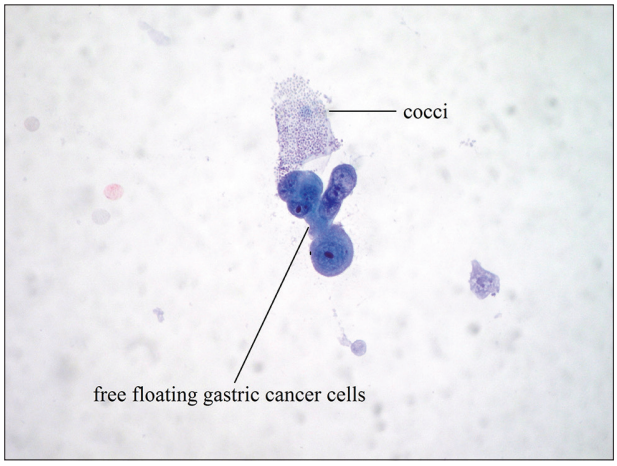

Figure 2: Gastric lavage gastric cancer cells with numerous cocci (Papanicolaou stain, 9100 oil immersion. Magnification: 44× field of view)

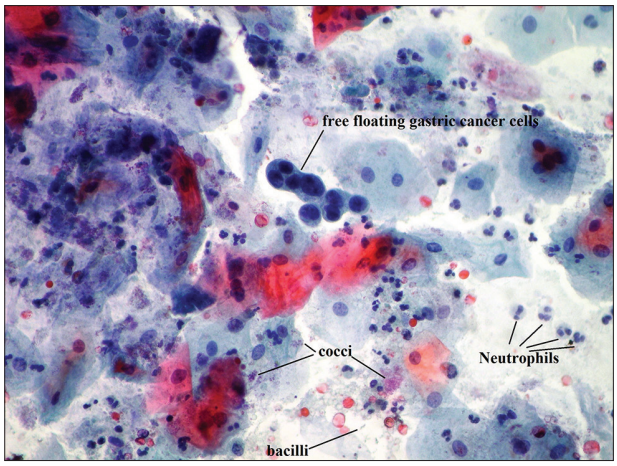

Figure 3: Malignant cells exfoliated into gastric lavage with cocci, bacilli, and neutrophils (Papanicolaou stain, 9100 oil immersion. Magnification: 44× field of view)

months (range: 2–77) of the 46 dead patients, 21 subjects were GL1 MB1, 2 GL1 MB0, 20 GL0 MB1, and 3 GL0 MB0; of the 33 alive subjects, 12 were GL1 MB1, 4 GL1 MB0, 12 GL0 MB1, and 5 GL0 MB0. The Kaplan-Meier model showed significant differences of OS between GL1 MB1 and GL1 MB0 groups (20.5 *vs* 45.8 months, respectively, $P = 0.049$) [Figure 4]. Precisely, in GL1 MB1 group, following surgery, there were 12 alive and 21 dead patients. For 7 alive patients, less than 10 months passed by from intervention. As for deaths, 10 occurred after 10 months from surgery, 13 after 20 months, 19 after 30 months, and 20 after 40 months. Concerning GL1 MB0 group, 2 deaths occurred after 19 and 24 months while 4 patients are still alive after 6, 38, 42, and 62 months. GL1 MB1 strongly correlated with advanced disease (T3-T4 with $P = 0.049$ and Stage 3-4 with $P = 0.035$) [Table 1]. At univariate analysis, the GL1 MB1 parameter resulted a significant prognostic factor for OS ($P = 0.008$) [Table 2]. Furthermore, multivariate analysis revealed GL1 MB1 as an independent prognostic factor of OS [$P = 0.013$ with an overall model fit of $P < 0.001$, Table 3]. In addition, GL1 MB1 significantly associated with the preoperative diagnosis of *H. pylori* infection [$P = 0.011$, Table 1].

Discussion

In the last decade, cytologic and molecular investigation of GL has provided interesting findings in terms of diagnosis, screening, prognosis, and treatment of GC patients.[7-17,28-30] Concerning the cytologic aspect, as suggested by numerous

Table 1: Clinicopathologic characteristics of the 79 gastric cancer patients related with the combined "gastric lavage cancer cells/microbiota" ("GL1/GL0 MB1/MB0") parameter

Clinicopathologic feature	Result	Association with GL1 MB1
Sex	M: 35 (44.3%); F: 44 (55.7%)	*P*=0.587
Age (mean years)	70.7 years (range: 42-88);	***P*=0.013**
	GL1 MB0: 61; GL1 MB1: 72	
Tumor Site	proximal*: 32 (33%); distal*: 47 (67%)	*P*=0.586
Siewert Type	Type 1 and 2: 12 (12.3%)	*P*=0.596
	Type 3 and non-Siewert cancers: 87	
NAT	18 (18.5%)	*P*=0.689
AT	30 (31%)	*P*=0.292
T	T category; T1: 17; T2: 18; T3: 12; T4: 32	*P*>0.05
	T3-T4: 44 (45%)	***P*=0.049**
N	N category	*P*=0.161
	N1: 13; N2: 14; N3: 26; N1-3: 53	*P*>0.05
M	M0: 81 (83.55%); M1: 16 (16.5%)	*P*=0.150
Stage	Category; 1: 24; 2: 15; 3: 23; 4: 18	*P* = > 0.05
	Stage 3-4: 39 (40%)	***P*=0.035**
G	G category; G1: 10; G2: 13; G3: 56	*P*>0.05
Lauren Classification	intestinal: 62 (64%); diffuse: 35 (36%)	*P*=0.236
WHO classification	WHO category; tubular: 28 (29%)	*P*>0.05
Signet Ring Cells	18 (18.5%); absence: 79 (81.5%)	*P*=0.221
LVI	LVI0: 52 (53%); LVI1: 45 (47%)	*P*=0.203
PnI	PnI0: 73 (75%); PnI1: 24 (25%)	*P*=0.781
LNR	Category; 1: 29; 2: 7; 3: 17; 1-3: 53	*P*>0.05
N° lymph nodes	GL1 MB0: 26.5; GL1 MB1: 24.8	*P*=0.745
Gastrectomy type	Distal: 43 (44.3%); Total: 24 (24.7%)	*P*=0.489
Operative time (min)	GL1 MB0: 201; GL1 MB1: 219	*P*=0.557
PLS (days)	GL1 MB0: 8.8; GL1 MB1: 14.4	*P*=0.272
Tumor size (mm)	GL1 MB0: 28.3; GL1 MB1: 48.5	*P*=0.127
Preoperative Anemia	47 (48.5%); absence: 50 (51.5%)	*P*=0.131
Postoperative Complications	14 (14%)	*P*=0.949
BMI	GL1 MB0: 26.2; GL1 MB1: 23.7	*P*=0.257
Microbiota species	Cocci: 77; Bacilli: 9; Mixed: 11	*P*=0.472
GL histiocytes	Presence: 6 (6%); Absence: 91 (94%)	*P*=0.534
GL hyphae/spores	Presence: 12; Absence 85 (88%)	*P*=0.482
Presurgery *Hp* biopsy	*Hp* presence: 5; Absence: 92 (95%)	***P*=0.011**

GL1/GL0: Presence/absence of free malignant cells exfoliated into gastric lavage samples; MB1/MB0: Presence/absence of bacterial microbiota in gastric lavage samples; *Proximal site: Cardio-fundic and gastric body carcinomas; distal site: antro-pyloric cancers; NAT: Neoadjuvant therapy; AT: Adjuvant therapy; LVI: Lymphovascular invasion; PnI: Perineural invasion; LNR: metastatic lymph node ratio; PLS:Postoperative length of stay; BMI: Body mass index; GL: Gastric lavage; *Hp: Helicobacter pylori*; *P* and association written in bold are statistically significant (<0.05)

authors, the oncologic value of GL derives from its privilege of collecting GC products released directly by the tumor avoiding hepatic clearance, a condition known under the name of Metastasis VI.[8,9,31] In the presence of a patent gastrointestinal tube, the exfoliation of malignant cells into the gastric lumen (Metastasis VI) strongly suggests the possibility that other cell elements have already migrated or infiltrated the surrounding tissue following the classical routes of metastasis (invasion through vascular or lymphatic channels, lymph nodes, direct contact, intraperitoneal or mesogastric seeding).[9] On the other hand, when obstruction by GC has occurred especially at cardia or pylorus, a number of cancerous cells, surviving for a long time due to a phenomenon called anoikis resistance, could colonize the gastric lumen, deposit on gastric or esophageal mucosa, and promote a metastasis.[11] Moreover, in most recent years, analysis of GL and stomach acid has been enriched with a further new perspective on GC research: the gastric bacterial MB.[18-25] Concerning the gastric microbial community, *H. pylori* infection indeed plays a pivotal role in GC carcinogenesis.[18-25,32,33] However, latest studies suggested that colonization of other non-*H. pylori* bacteria in the stomach (such as *Propionibacterium acnes*, *Prevotella copri*, and *Eubacterium cylindroides*) can also stimulate GC risk by producing proinflammatory cytokines such as IL 15 and lymphocytic gastritis.[34-37] Taking a cue from such new literature data, for this study, we

Table 2: Univariate analysis of significant prognostic factors for overall survival

Variable	*P*	Variable	*P*
GL1 MB1	**0.008**	**LVI**	**0.013**
Stage	**0.006**	**PnI**	**<0.001**
Stage 3C	**0.049**	**N**	**0.014**
Stage 3-4	**0.048**	**N2**	**0.032**
Stage 4	**0.001**	**N3**	**0.004**
Lauren type	**0.041**	**PnI**	**<0.001**
LNR3	**0.004**	**LNR**	**0.040**
M	**0.003**	**Size**	**0.044**
NAT	**0.010**	**Curative surgery**	**0.010**
Preoperative anemia	**0.021**	**T3-T4**	**0.040**
R	**<0.001**	**T4**	**0.026**
Others	>0.05	Others	>0.05

GL1 MB1: Intragastric copresence of cancer cells and bacterial microbiota; LNR: Metastatic lymph node ratio; LVI: Lymphovascular invasion; PnI: Perineural invasion; NAT: Neoadjuvant therapy; variables and *P* written in bold are statistically significant (<0.05)

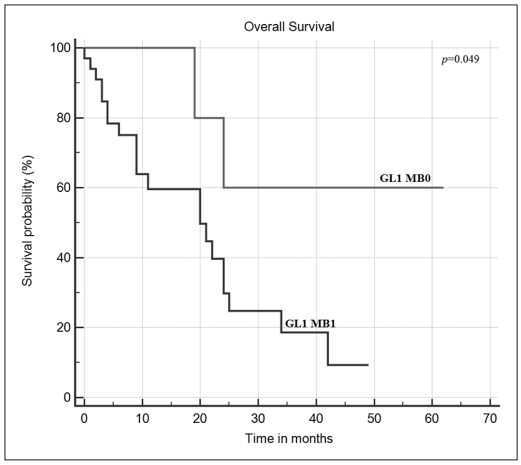

Figure 4: Difference of survivals between patients with intragastric copresence of cancer cells and microbiota (GL1 MB1) and subjects with exfoliated malignant cells but without microbiota (GL1 MB0)

Table 3: Multivariate analysis of independent prognostic factors for overall survival

Independent variables	b	SE	Wald	*P*	Exp(b)	95% CI of Exp(b)
GL1 MB1	4.9254	1.9995	6.0676	**0.0138**	137.7445	2.7354 to 6936.2278
T3-T4	-20.8581	10.3869	4.0326	**0.0446**	8.7384E-010	1.2588E-018 to 0.60
T4	11.3276	4.8025	5.5633	**0.0183**	83086.3574	6.7845 to 1.02E+009
N2	0.4916	2.0694	0.05643	0.8122	1.6349	0.0283 to 94.4130
N3	-13.6155	9.0095	2.2838	0.1307	0.0000	2.6169E-014 to 57.0
Stage 3-4	9.6674	7.2151	1.7953	0.1803	15793.8721	0.0114 to 21.9E+009
Stage 4	-18.6568	10.3357	3.2583	0.0711	7.8967E-009	1.2575E-017 to 4.95
LVI	-1.8558	1.1352	2.6726	0.1021	0.1563	0.0169 to 1.4465
PnI	10.9953	4.7423	5.3758	**0.0204**	59593.7547	5.4764 to 648492966
Lauren type	4.8801	2.7423	3.2076	0.0733	131.6499	0.6309 to 27471.123
LNR3	10.5443	6.4439	2.6776	0.1018	37961.2555	0.1242 to 11.6E+009
M	17.9615	8.8487	4.1203	**0.0424**	63178227.38	1.8553 to 2.1514E+0
NAT	10.5263	6.0707	3.0066	0.0829	37282.7020	0.2535 to 5.48E+009
Curative surgery	5.3211	5.9040	0.8123	0.3675	204.5997	0.0019 to 21703212
PO NS complications	4.1918	1.7152	5.9727	**0.0145**	66.1412	2.2933 to 1907.5679
Preoperative anemia	4.2286	1.5673	7.2792	**0.0070**	68.6244	3.1794 to 1481.1931
R	15.8072	7.5381	4.3973	**0.0360**	7327999.730	2.8081 to 19.1E+012
Overall model fit: *P*<0.0001						

b: Regression coefficient beta; SE: Standard error; Wald: b/SE2; Exp (b): Exponentiation of the beta coefficient; CI: Confidence interval; LNR: Metastatic lymph node ratio; NAT: Neoadjuvant therapy; NS: Non-surgical; variables and *P* written in bold are statistically significant (<0.05)

wanted to enrich our previously reported line of research (the cytopathologic analysis of GL from GC patients) by combining it with examination of intragastric MB: as a consequence, we assessed the cyto-microbiologic parameter of "GL MB." In our patient population, analysis of this character provided original and interesting results. In fact, subjects showing GL1 and MB1 had poorer survival compared with GL1 MB0 group (20.5 *vs* 45.8 months, respectively, $P = 0.049$) [Figure 4]; such a result could confirm a pro-tumorigenic role of some gastric microbiota as suggested by previous studies.[34-36] This is also corroborated by the fact that, in our series, MB1 in conjunction with GL1 strongly correlated with tumor aggressiveness in advanced phase of disease (T3-T4 with $P = 0.049$ and Stage 3-4 with $P = 0.035$) [Table 1]. Furthermore, the GL1 MB1 parameter resulted a significant prognostic factor for OS in univariate analysis ($P = 0.008$, Table 2) and an independent prognostic factor of OS at multivariate analysis ($P = 0.013$ with an overall model fit of $P < 0.001$, Table 3). In other words, the absence of bacterial microbiota (MB0) in GL1 GC patients seemed to be a protective factor. In addition, GL1 MB1 was significantly associated with the preoperative diagnosis of *H. pylori* infection ($P = 0.011$, Table 1).

In the light of our results, the mixed cyto-microbiological test on GL seems quite interesting to perform in GC patients, especially from a prognostic and therapeutic point of view. Our findings, in fact, strengthening the carcinogenic role executed by Metastasis VI and *H. pylori* but also suggesting the cooperation between such features and the other non-*H. pylori* pro-oncogenic germs within the endogastric microenvironment, showed that GL1 MB1 GC patients had a poorer OS in comparison with GL1 MB0 GC subjects.[18-25,32-36] In this regard, the treatment of non-*H. pylori* bacteria could exert a conspicuous benefit for individuals with related precancerous gastric lesions (such as lymphocytic gastritis), just as already proven by antibiotic therapy for *H. pylori* infection.[30,37] Further studies dealing with GC patients showing malignant endogastric exfoliation in combination with intragastric microbiota (GL1 MB1 GC subjects) are needed to corroborate our data.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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