A multicenter prospective study of first-line antibiotic therapy for early-stage gastric mucosa-associated lymphoid tissue lymphoma and diffuse large B-cell lymphoma with histological evidence of mucosa-associated lymphoid tissue

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Supplementary Appendix Contents

Supplementary Methods

Histological diagnosis of mucosa-associated lymphoid tissue (MALT) lymphoma and diffuse large B-cell lymphoma with MALT

Lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) and diffuse large B-cell lymphoma (DLBCL) with accompanying MALT lymphoma (DLBCL[MALT]) were diagnosed by histopathologists at individual hospitals, in accordance with specified criteria.[1-4] Each diagnosis was reviewed by members of the Taiwan Cooperative Oncology Group Pathology Committee, as described previously.[5] MALT lymphoma was diagnosed according to the criteria of the 2008 World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues[1,2] and characterized by the presence of small-to-medium-sized lymphoid cell infiltrates (these cells contained slightly irregular nuclei with moderately dispersed chromatin and inconspicuous nucleoli resembling centrocytes); presence of lymphoepithelial lesions (LELs) in the epithelium of gastric glandular tissues, invaded and destroyed by discrete aggregates of lymphoma cells; and absence of confluent clusters or sheets of large cells resembling centroblasts or lymphoblasts.[1-4] To exclude *Helicobacter pylori* (HP) -reactive inflammatory process-like lymphoma cells, clonality

analyses of immunoglobulin H gene rearrangement in B-cell lymphoma were performed in patients whose tumors lacked LELs.[1,2,4]

A diagnosis of DLBCL components in DLBCL(MALT) was made according to the histological criteria described by 2008 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues,[1,2] based on the presence of a diffuse increase in large cells or solid and sheet-like large cells resembling centroblasts or lymphoblasts. Occasional clusters of transformed blast cells or sheets of transformed blast cells (up to 20 cells without the formation of larger sheets) confined within the colonized follicles were considered an immune response to HP stimulation rather than DLBCL(MALT).[1,4,6] To avoid the misinterpretation of clusters of reactive germinal center cells from overrun follicles as areas of DLBCL components, additional immunohistochemical (IHC) staining was performed for Ki-67 (a protein involved in cell proliferation), germinal center cell markers (cluster of differentiation [CD]10 and BCL6), and dendritic cell markers (CD21) in these large cells.[1,2,4] If these large cells exhibited a higher Ki-67 labeling index and absence of CD10, BCL6, and CD21, these large cells were regarded as DLBCL, whereas large cells with a lower Ki-67 labeling index that expressed CD10, BCL6, or CD21, were classified as clusters of germinal center cells, not DLBCL.[1,2] In addition, IHC staining for cytokeratin (1:50, clone

AE1/AE3; Ylem, Rome, Italy) was performed to identify LELs in the context of the minimal MALT lymphoma components in DLBCL.[7]

Staging work-up, diagnosis of *Helicobacter pylori* infection, and *Helicobacter pylori* eradication regimens

Patients were required to have evaluable disease by endoscopy, with nodal status assessed by computed tomography (CT) scan. Endoscopic ultrasonography (EUS) was optional for evaluating the depth of tumor infiltration and perigastric lymph node enlargement, because EUS facility was not available at some of the participating institutions and previous studies suggested that advances in radiographic techniques improved the accuracy of gastric cancer T-staging by CT scan, with accuracies of 71.4–82.6% by CT scan and 65–92.1% by EUS.[8] Staging workup included a detailed physical examination; inspection of Waldeyer's ring; CT scan of the neck, chest and abdomen; a small bowel series; barium enema study of the colon and rectum; and bone marrow aspiration and biopsy. Tumors were staged based on CT findings, according to Musshoff's modification of the Ann Arbor staging system, with stage IE tumors confined to the wall of the stomach and stage IIE1 tumors showing perigastric lymph node involvement. The presence of HP infection was

defined as positive results on biopsy, histology, a urease test, a ^{13}C urea breath test, serology, or culture. HP-positive patients who agreed to receive the first-line HP eradication (HPE) regimen, consisting of 500 mg amoxicillin administered four times a day, 500 mg clarithromycin administered twice a day, and 20 mg omeprazole administered twice a day for two weeks, were enrolled in this prospective study (**Online Supplementary Figure S1**).

Clinical follow-up and histological evaluation

Patients were scheduled for the first follow-up upper gastrointestinal endoscopic examination 4 weeks after completion of HPE and were followed-up every 3 months thereafter until complete remission (CR) was achieved or treatment failed (**Online Supplementary Figure S1**). At each follow-up endoscopic examination, four to six biopsy specimens from the antrum and body of the stomach were evaluated for HP infection, and a minimum of six biopsy samples from each tumor and suspicious area were histologically evaluated. After HP treatment, HP infection status was determined by histological examination, biopsy urease test, and ^{13}C urea breath test.

Because this study started in 2006, patients' histological features were initially evaluated using the scoring system described by Wotherspoon et al.[9]

At present, however, the histological scoring system of the Groupe d'Etude des Lymphomes de l'Adult (GELA) is recommended to improve the consistency of findings of histologic regression after first-line HPE for gastric MALT lymphoma.[10,11] Therefore, the responses of patients with MALT lymphoma and DLBCL(MALT) of stomach who completed HPE and had successful HPE were reassessed using the GELA scoring system. [10,11]

CR was defined as the total disappearance of gross lymphoma and a negative histological finding (CR or probable minimal residual disease [pMRD]), whereas partial remission (PR) was defined as normalization or reduction of macroscopic findings, histological signs of lymphoma regression, and no signs of progression.[10,11] Stable disease (SD) was defined as unchanged macroscopic and/or histological findings, and progressive disease (PD) was defined by worsening of macroscopic findings, dissemination of gastric MALT lymphoma or DLBCL(MALT), or transformation into DLBCL.[10,11] Patients with gastric MALT lymphoma who had PD were removed from the study protocol, with their further treatment at the discretion of the physician in charge. Patients with gastric DLBCL(MALT) who failed treatment, defined as SD or PD on follow-up endoscopic examination and histological evaluation, were also removed from the study protocol and referred immediately for systemic chemotherapy.

Assessment of the t(11;18)(q21;q21) translocation in lymphoma cells

The presence of t(11;18)(q21;q21) was assessed by multiplex reverse transcription polymerase chain reaction followed by sequencing of the BIRC3-MALT1 fusion transcripts or by the interphase fluorescence in situ hybridization method using a commercially available probe (BIRC3/MALT1 dual-color, dual-fusion translocation probe; Vysis LSI/Abbott), with all assays performed according to the manufacturers' instructions.[12,13] Gastric MALT lymphoma samples with BIRC3-MALT1 fusion transcripts served as positive controls.

Immunohistochemistry for detection of BCL10, NF-κB, and cytotoxin-associated gene A (CagA)

Paraffin-embedded sections of pre-HPE endoscopic biopsies were assayed immunohistochemically using antibodies directed against BCL10 (sc-9560; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB (p65; sc-109; Santa Cruz Biotechnology), and CagA (A10; sc-28368, Santa Cruz Biotechnology), using an indirect immunoperoxidase method as described by the manufacturer.[6,13,14] Cell blocks of a CagA-translocated human B-cell line served as a CagA-positive control. To confirm specificity, staining was performed on paraffin-embedded sections in the absence of the first and/or

second primary antibodies as negative controls.

The percentages of positive cells were averaged to yield immunohistological scores of 0%–100%. Staining for BCL10 and NF-κB (p65) was considered positive if the protein was detected in more than 10% of the tumor cell nuclei.[6,14] Positive expression of the CagA marker was defined as moderate or strong immunostaining of more than 10% of the cells (tumor cells with readily visible brown staining distinctly marking the tumor cell nucleus and cytoplasm), as described.[13,14]

Double-color IHC analysis was performed using multiplex-IHC reagents (Biocare Medical, Concord, CA, USA). All steps prior to primary antibody incubation were performed as mentioned above. Sections were incubated with primary antibodies against CagA (A10; sc-28368, Santa Cruz Biotechnology) and CD20 (EP459Y; ab78237, Abcam, Cambridge, MA) simultaneously, followed by use of the MACH1 universal HRP-polymer detection Kit (Biocare Medical, Concord, CA, USA). CagA and CD20 expression was detected using chromogens, including 3,3′-diaminobenzidine and vina green (Vina Green™ Chromogen Kit, Biocare Medical, Concord, CA, USA).

Statistical analysis

The primary endpoint was the therapeutic effectiveness (CR) of first-line HPE in stage IE/IIE1 primary MALT lymphoma and DLBCL(MALT) of the stomach. The secondary endpoints were (1) the efficacy of HPE, as shown by the duration of CR, (2) the relationship between CR and disease stage (i.e. stages IE and IIE1), and (3) the usefulness of CagA expression, nuclear expression of BCL10 and NF-κB (p65), and the presence of the t(11;18)(q21;q21) translocation in predicting the HP-independence (the lack of complete lymphoma regression after HPE) of stage IE/IIE1 primary MALT lymphoma and DLBCL(MALT) of the stomach.

Categorical variables were compared using Fisher's exact test, whereas continuous variables were compared using Nonparametric Wilcoxon Rank Sum test. Follow-up data that became available on June 30, 2018, were analyzed. Time to CR of HP-dependent patients (the presence of complete lymphoma regression after HPE), calculated from the completion of antibiotic therapy to first evidence of CR by Kaplan–Meier analysis. The duration of overall survival (OS) was defined as the interval from the date of registration to death from any cause; the duration of relapse-free survival (RFS) was defined as the interval from the date of first documentation of CR to the date of histological

confirmation of recurrence in HP-dependent cases. RFS and OS were estimated by the Kaplan–Meier method and compared by log-rank tests. Multivariate analyses were performed using a logistic regression model for tumor response to HPE (factors with $P<0.05$). All statistical tests were two-sided, with P values less than 0.05 considered statistically significant.

Supplementary Results

Clinicopathological characteristics of patients

There were no statistically significant differences between the MALT lymphoma and DLBCL(MALT) groups in gender, sex, performance status, duration of symptoms, lactate dehydrogenase (LDH) level, endoscopic appearance, lesion site, depth of involvement, and tumor stage. However, β2-microglobulin concentration was significantly higher in the DLBCL(MALT) than in the MALT lymphoma group (*P*=0.0197) (**Table 1**). The percentages of patients previously treated for HP infection, and those with peptic ulcer disease, hematemesis or melena, prior history of other malignancies, and symptomatic disease did not differ in the two patient groups (**Online Supplementary Table S1**), nor did, the expression of CagA and the nuclear expression of BCL10 and NF-κB (p65) (**Table 1**). However, t(11;18)(q21;q21) translocations were observed only in four (11%) of the 36 patients with MALT lymphoma, but not in any patient with DLBCL(MALT) (**Table 1**).

Follow-up and salvage treatment for patients non-responsive to first-line *Helicobacter pylori* eradication therapy

The two patients in the MALT lymphoma group experienced local lymphoma recurrence after 12 months and 24 months of CR, respectively. The first patient showed HP re-infection at the time of relapse, whereas the second showed evidence of HP re-infection 6 months before histological evidence of tumor recurrence. The HP re-infections and the relapsed tumors were responsive to second- or third-line antibiotic therapy.

Of the 12 patients who did not respond to HPE (HP-independent tumors), two, both with DLBCL(MALT), were treated with rituximab-based systemic chemotherapy, with one treated with rituximab-CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) and the other with R-COP, with all 2 achieving CR after systemic chemotherapy. Of the 10 patients with HP-independent gastric MALT lymphoma, eight were treated with the oral alkylating agent, chlorambucil; one received involved-sites radiotherapy, with 34 Gy divided into 17 fractions; and one was monitored without treatment. Of the 11 patients who received salvage treatment after their tumors achieved SD or PD, nine, two with DLBCL(MALT) and seven with gastric MALT lymphoma, achieved CR after second-line treatment. The other two patients, who did not respond to chlorambucil, subsequently responded to rituximab monotherapy.

Correlation of clinicopathological features, expression of CagA, BCL10 and NF-κB (p65), and t(11;18)(q21;q21) with tumor response to first-line *Helicobacter pylori* eradication therapy

There were no statistically significant differences in age, gender, performance status, symptom duration, LDH level, β2-microglobulin level, endoscopic appearance, and depth of involvement by EUS between patients with HP-dependent and HP-independent tumors, except distal lesions were closely associated with the HP dependence (*P*=0.0242). CR rates were similar in patients with stage IE (75.0% [30/40]) and stage IIE1 (66.7% [4/6]) disease (*P*=0.6435). Of the four patients with stage IIE1 disease who achieved CR, two each were diagnosed with DLBCL(MALT) and MALT lymphoma.

The frequency of nuclear BCL10 expression was significantly higher in HP-independent (83.3% [10/12]) than in HP-dependent tumors (8.8% [3/34]) (*P*<0.001). Similarly, nuclear NF-κB (p65) expression was detected in nine (75.0%) of 12 HP-independent and in four (11.8%) of 34 HP-dependent tumors (*P*=0.0001). Nuclear BCL10 were closely associated with nuclear NF-κB (p65) expression in these tumors (*P*<0.001). The t(11;18)(q21;q21) translocation was detected in four (33.3%) of 12 HP-independent and in 0 (0%) of 34 HP-dependent tumors (*P*=0.003). All four tumors with the t(11;18)(q21;q21)

translocation were positive for nuclear BCL10 and nuclear NF-κB (p65) expression. CagA expression was detected in 33 (71.7%) of the 46 patients, with 10 and 23 having scores of 2 and 3, respectively. In contrast, 13 patients (28.3%) were negative for CagA, including 11 and two with scores of 0 and 1, respectively. The frequency of CagA expression was significantly higher in HP-dependent (85.3% [29/34]) than in HP-independent (33.3% [4/12]) tumors (*P*=0.001).

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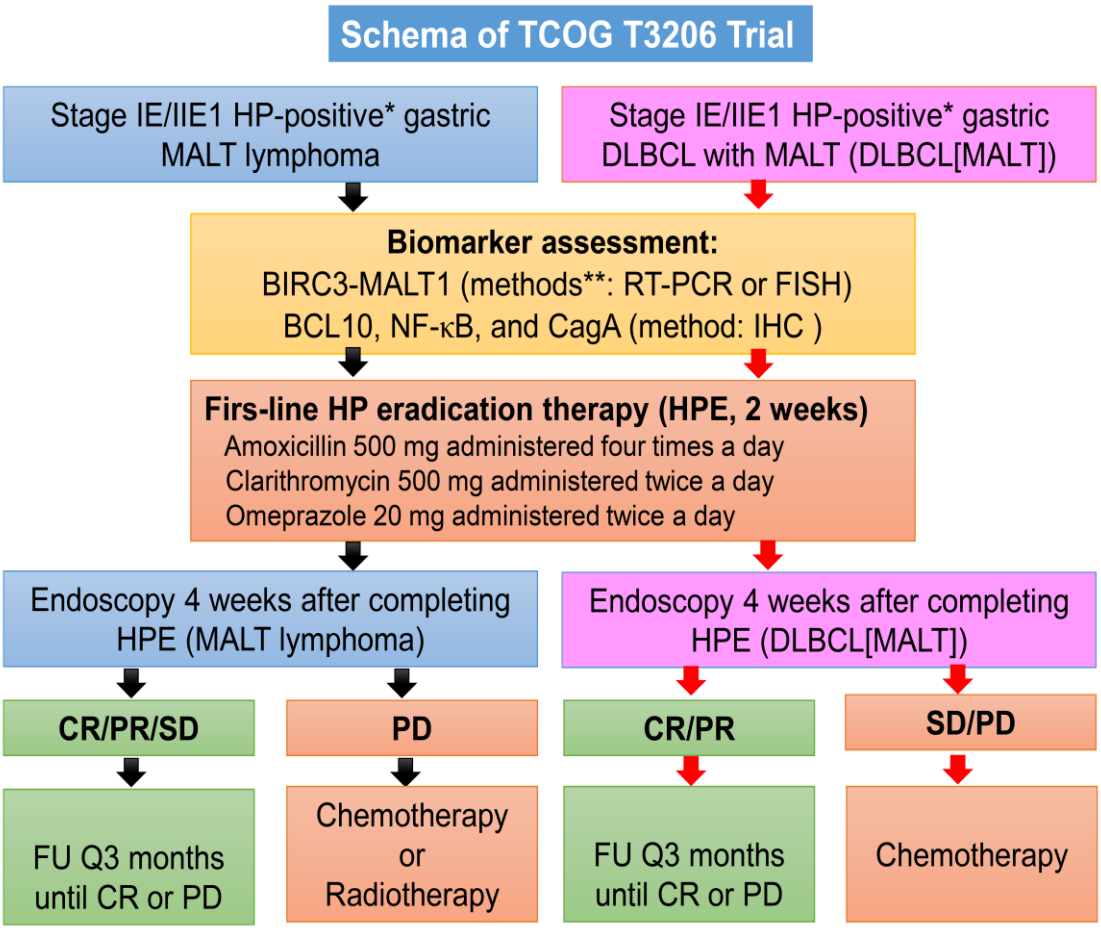
Supplementary Table S1. Demographics and Baseline Characteristics of Patients Stratified by Histological Classification

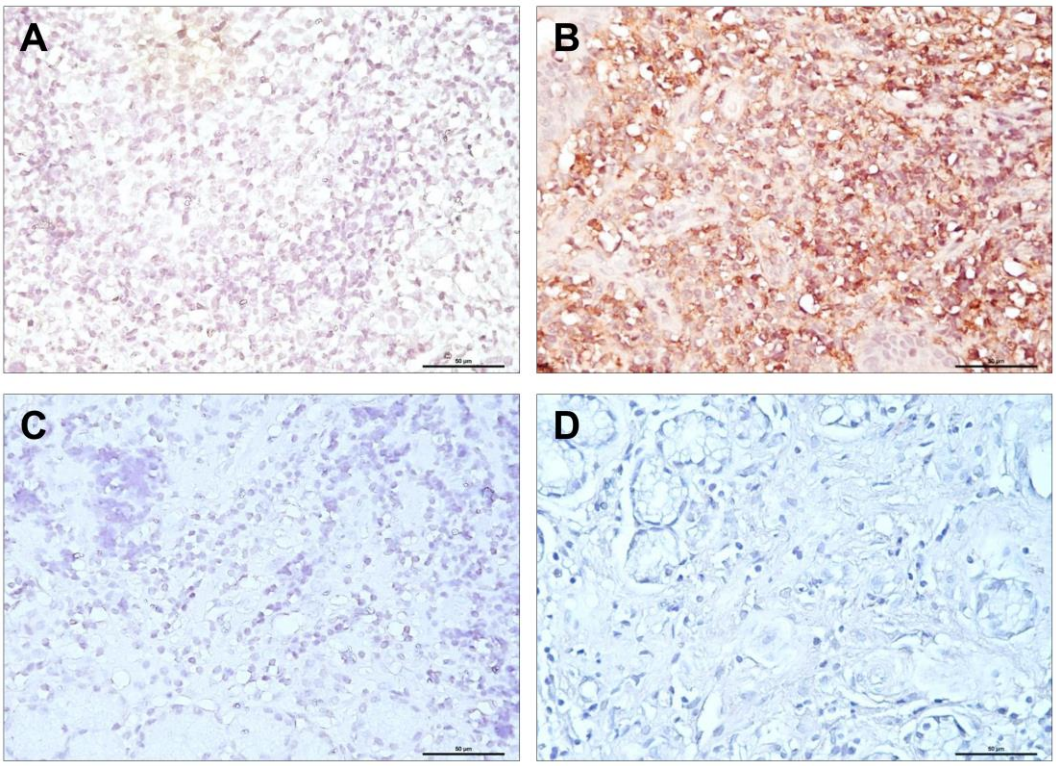
Variables	Total (N = 46)		MALToma (N = 36)		DLBCL(MALT) (N = 10)		*P* value
Patient ever treated by anti-HP therapy							1.0000
No	40	(86.96%)	31	(86.11%)	9	(90.00%)	
Yes	6	(13.04%)	5	(13.89%)	1	(10.00%)	
Patient ever treated as peptic ulcer disease							1.0000
No	30	(65.22%)	23	(63.89%)	7	(70.00%)	
Yes	16	(34.78%)	13	(36.11%)	3	(30.00%)	
Prior history of hematemesis and/or melena							0.5296
No	43	(93.48%)	34	(94.44%)	9	(90.00%)	
Yes	3	(6.52%)	2	(5.56%)	1	(10.00%)	
Symptomatic stage							0.5975
Asymptomatic	40	(86.96%)	32	(88.89%)	8	(80.00%)	
Symptomatic	6	(13.04%)	4	(11.11%)	2	(20.00%)	
Prior history of other malignancy							0.3913
No	44	(95.65%)	35	(97.22%)	9	(90.00%)	
Yes	2	(4.35%)	1	(2.78%)	1	(10.00%)	
Chronic HCV infection							0.0609
No	41	(89.13%)	34	(94.44%)	7	(70.00%)	
Unknown	5	(10.87%)	2	(5.56%)	3	(30.00%)	
HP eradication after protocol treatment							0.1316
Yes	31	(67.39%)	22	(61.11%)	9	(90.00%)	
No	15	(32.61%)	14	(38.89%)	1	(10.00%)	

Abbreviation: N, number; MALToma, mucosa-associated lymphoid tissue lymphoma; DLBCL(MALT), diffuse large B-cell lymphoma with MALToma; HP, *Helicobacter pylori*;

* *P* vales were calculated with Fisher's exact test for categorical variables, and with Nonparametric Wilcoxon Rank Sum test for continuous variables.



Supplementary Figure S1. Scheme of the protocol of first-line *Helicobacter pylori* eradication therapy for early-stage *Helicobacter pylori*-positive gastric MALT lymphoma and gastric DLBCL(MALT) (Taiwan Cooperative Oncology Group (TCOG) T3206 trial). *The presence of *Helicobacter pylori* (HP) infection was defined as positive results on biopsy, histology, a urease test, a ^{13}C urea breath test, serology, or culture). **. The presence of t(11;18)(q21;q21) was assessed by multiplex reverse transcription polymerase chain reaction (RT-PCR) followed by sequencing of the BIRC3-MALT1 fusion transcripts or by the interphase fluorescence in situ hybridization (FISH) method using a commercially available probe (*BIRC3/MALT1* dual-color, dual-fusion translocation probe; Vysis LSI/Abbott). The expression of BCL10, NF-κB, and CagA was assessed by immunohistochemistry (IHC). Abbreviation: CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease.



Supplementary Figure S2. Changes in the expression levels of CagA in serial tumor cells before and after completion of *Helicobacter pylori* eradication therapy (A) Moderate baseline expression of CagA in case #3 tumor cells. (B) A high baseline expression of p-SHP-2 in case #3 tumor cells. (C) CagA expression was no longer detectable in remitting tumor cells 1 month after completion of *Helicobacter pylori* eradication (HPE) (D) CagA expression was not detectable in gastric biopsies 4 months after completion of HPE (case #3, the time to CR after completion of HPE was 4 month)