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Association between *Helicobacter suis* infection and the pathogenesis of gastric mucosa-associated lymphoid tissue lymphoma

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ABSTRACT

Objective: In patients with *Helicobacter pylori*-negative gastric mucosa-associated lymphoid tissue (MALT) lymphoma, eradication therapy is only effective in some cases, suggesting that infections with non-*H. pylori Helicobacter* (NHPH) species may also be involved. Therefore, we examined the prevalence of infection with *Helicobacter suis*, an NHPH, in gastric MALT lymphoma patients.

Methods: We examined the *H. suis* infection status of 15 patients with *H. pylori*-negative gastric MALT lymphoma. To examine the infection prevalence through species-specific PCR analysis, DNA was extracted from the lymphoma lesion and the background gastric mucosa. We compared the PCR products amplified from DNA extracted from different lesions.

Results: *H. suis* was detected in 3 of the 15 cases (20.0%), but only in the background gastric mucosa and not in the lymphoma lesion. *H. suis* was completely eradicated in all three cases and two of the three cases exhibited complete remission of the lymphoma.

Conclusion: These results indicated that *H. suis* infection may be involved in the pathogenesis of gastric MALT lymphoma. We conclude that it is desirable to use multiple biopsies, including the background mucosa, to evaluate NHPH infection in gastric MALT lymphoma patients.

Key words: *Helicobacter suis*, *Helicobacter pylori*, *mucosa-associated lymphoid tissue (MALT) lymphoma*, *non-Helicobacter pylori Helicobacter (NHPH)*

INTRODUCTION

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma is an extranodal B cell lymphoma characterized by morphologically diverse cells infiltrating into the mucosa-associated lymphoid tissues, mainly in the follicular marginal zone[7]. Gastric MALT lymphoma is thought to develop due to chronic inflammation caused by *Helicobacter pylori* infection. Generally, in *H. pylori*-positive gastric MALT lymphoma cases, *H. pylori* eradication therapy leads to complete regression of the lymphoma[13,14,17]. However, in some cases, even in patients without *H. pylori* infection, the eradication treatment has been reported to successfully result in complete remission (CR)[10–12].

One hypothesis for this phenomenon is that non-*H. pylori Helicobacter* (NHPH) infections may also be involved in the pathogenesis of gastric MALT lymphoma[13]. NHPHs are also referred to as *Helicobacter heilmannii*-like organisms[3] or *H. heilmannii* sensu lato. Previous studies have shown that infections with NHPH lead to various gastric diseases, including gastric MALT lymphoma[5,9]. The prevalence of NHPH infections ranges between 0.2–6% in the general population, which is much lower than the prevalence of *H. pylori* infections[15,16,18]. *Helicobacter suis* is an NHPH that has been recognized as a new taxonomic group of *Helicobacter* species and it may be the NHPH that has the strongest relevance to the development of gastric MALT lymphoma[1,3,6,8,16].

Because the prevalence of *H. suis* infection is higher in *H. pylori*-negative patients than in *H. pylori*-positive patients[8], we randomly selected patients with *H. pylori*-negative MALT lymphoma. We examined the prevalence of *H. suis* infection in 15 *H. pylori*-negative gastric MALT lymphoma patients and compared the PCR results of DNA extracted from various sites (lymphoma lesion and background gastric mucosa).

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Table 1 Characteristics of *H. pylori*-negative gastric MALT lymphoma patients

Patients	Age (yr)	Sex	*H. suis* infection	Endoscopic Appearance	Gastric atrophy	Lesion site	Response to eradication therapy
1	61	Male	–	Superficial	C-0	Corpus	NC
2	68	Female	–	Superficial	C-1	Corpus	NC
3	56	Female	–	Superficial	C-0	Corpus	NC
4	44	Male	+	Superficial	C-0	Corpus-Fundus	CR
5	73	Female	–	Superficial	C-0	Fundus	NC
6	59	Female	–	Superficial	C-0	Corpus	CR
7	70	Female	+	Superficial	C-0	Corpus	NC
8	67	Female	–	Superficial	C-0	Corpus	NC
9	65	Female	–	Superficial	C-0	Corpus	NC
10	32	Male	+	Superficial	C-1	Corpus	CR
11	59	Female	–	Superficial	C-0	Fundus	CR
12	66	Female	–	Superficial	C-1	Fundus	CR
13	50	Male	–	Superficial	C-1	Corpus	CR
14	73	Female	–	Superficial	O-3	Angle	CR
15	60	Male	–	Superficial	C-0	Corpus	CR

CR: complete remission, NC: no change

MATERIALS AND METHODS

Patients

Fifteen *H. pylori*-negative, *API2-MALT1 translocation*-negative gastric MALT lymphoma patients were randomly selected for this study. They were diagnosed with gastric MALT lymphoma according to the diagnostic criteria of the World Health Organization and symptoms were consistent with grade 4 or 5 of the Wotherspoon histological scoring system. Patients were treated and monitored at the Hiroshima University Hospital between 2001 and 2017. After treatment, histopathological evaluation was performed using the Group d'Etude des Lymphomes deI'Adulte (GELA) grading system. CR of lymphoma was defined as complete histological response (ChR) or probable minimal residual disease (pMRD) in the GELA system, or grades 0 or 1 in the Wotherspoon scoring system. *H. pylori* infection was determined according to histological analysis, an anti-*H. pylori* IgG assay (E-plate, Eiken, Tokyo, Japan; cut-off level, 10 U/mL), a urea breath test (Otsuka, Tokushima, Japan; cut-off level, 2.5‰), and species-specific PCR analysis. Patients were considered *H. pylori*-positive if at least one of the tests yielded a positive result, and *H. pylori*-negative if all tests were negative. Endoscopic biopsy specimens were obtained from lymphoma lesions and the background gastric mucosa. One biopsy specimen from the lymphoma lesion was immediately frozen and stored until subsequent PCR analysis (fresh frozen [FF] tissues). Formalin-fixed paraffin-embedded (FFPE) specimens were used for histopathological diagnosis of MALT lymphoma and analysis of the background gastric mucosa. The *API2-MALT1* chimeric gene was detected by RT-PCR and/or fluorescence *in situ* hybridization. The patients had a mean age of 60.1 years and included 5 males and 10 females (Table 1). These studies were performed in accordance with the Declaration of Helsinki and were approved by the Institutional Review Board of the Hiroshima University Hospital. Informed consent was obtained from all patients.

DNA extraction and PCR

The All Prep™ DNA/RNA Micro Kit (Qiagen, Hilden, Germany) and the Gene Read™ DNA FFPE Kit (Qiagen) were used to extract DNA from FF and FFPE samples, respectively, according to the manufacturer's instructions. Detection of *H. suis* and *H. pylori* was performed through species-specific PCR. DNA integrity was confirmed by the amplification of the human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The PCR conditions, thermal cycler parameters, and gene-specific primers used for amplification are listed in Table 2.

As positive controls, we used specific DNA amplicons that had previously been validated under the same conditions. For negative controls, RNAase-free water was added instead of DNA template. PCR products were separated by electrophoresis through a 2.0% agarose gel and were then stained with ethidium bromide.

RESULTS

Detection of *H. suis* infection by species-specific PCR

To evaluate *H. pylori* and *H. suis* infection status, we performed *H. pylori* species-specific PCR using DNA extracted from FFPE and FF tissues. *GAPDH*-specific bands were amplified and detected in all cases. Therefore, DNA integrity was well maintained in both FFPE and FF tissues. *H. pylori*-specific bands were not detected in any of the patients, indicating that these were indeed *H. pylori*-negative lymphoma cases. We then determined the *H. suis* infection status of *H. pylori*-negative gastric MALT lymphoma patients using species-specific PCR. *H. suis*-specific bands were detected in 3 of the 15 patients (20.0%). Specific amplicons were only

Table 2 PCR conditions, thermal cycling parameters, and gene-specific primers used for amplification

Markers	Oligonucleotides 5'-3'	Size of amplicon	Thermal cycler condition	References
H. suis (*ureA*)	F: CACCACCCCGGGGAAGTGATCTTG R: CTACATCAATCAAATGCACGGTTTTTTCTTCG	253 bp	95°C for 7 min 94°C for 30 sec 61°C for 40 cycles 72°C for 30 sec 72°C for 7 min	[8]
H. pylori (*ureAB*)	F: AAAGAGCGTGGTTTTCATGGCG R: GGGTTTTACCGCCACCGAATTTAA	217 bp	95°C for 7 min 94°C for 30 sec 60°C for 30 cycles 72°C for 30 sec 72°C for 7 min	[8]
Human GAPDH	F: CCAGGAGTGAGTGGAAGACA R: GCAAATGAGCCTACAGCAGA	130 bp	95°C for 7 min 94°C for 30 sec 60°C for 30 cycles 72°C for 30 sec 72°C for 7 min	This study

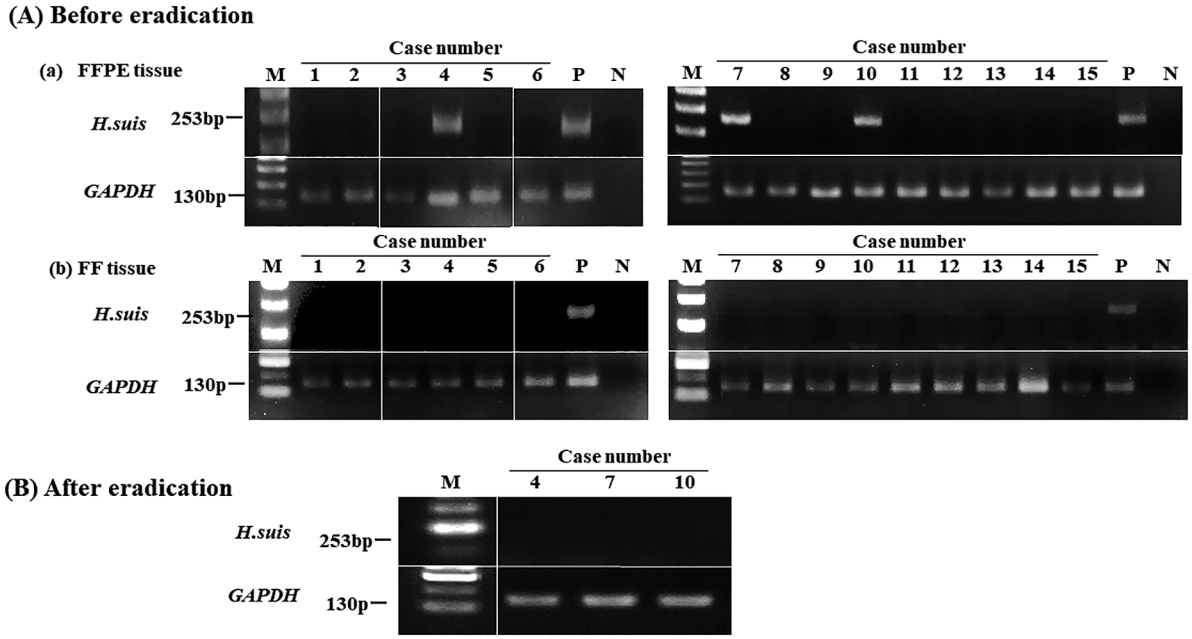

Figure 1 Detection of *H. suis* through species-specific PCR analysis. In 3 of the 15 patients, *H. suis*-specific bands were detected by PCR analysis of DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues (background gastric mucosa), but not DNA from fresh frozen (FF) tissues (tumor site) (A). *GAPDH* amplification confirmed the integrity of DNA from both FFPE and FF tissues. After eradication therapy, *H. suis*-specific bands were no longer detected (B). P and N are the positive and negative controls (water), respectively. Lane M, size marker.

detected in FFPE specimens (non-tumorous gastric mucosa), and not in FF specimens (tumor tissues).

To eradicate *Helicobacter* infection, these patients underwent treatment with orally administered lansoprazole (30 mg/day), amoxicillin (750 mg/day), and clarithromycin (400 mg/day) twice a day for 1 week. For all three patients, eradication of the *H. suis* infection was confirmed by PCR analysis. In addition, 2 of the 3 *H. suis*-positive lymphoma cases showed CR after the eradication treatment.

Case reports of *H. suis*-positive gastric MALT lymphoma patients treated with eradication therapy

The clinicopathological characteristics of *H. suis*-positive MALT lymphoma patients are summarized in Table 2. Immunoglobulin heavy chain (*IGH*) gene rearrangement was evaluated, and monoclonal *IGH* rearrangement was confirmed in all cases (data not shown). *API2-MALT1* chimeric transcripts were not detected in any of these cases (data not shown). We show a representative case of a 32-year-old male (case number 10). Endoscopic examination revealed a superficial elevated lesion (20 mm in diameter) on the angular incisures of the lesser curvature of the stomach (Figure 2A). Indigo carmine chromoendoscopy revealed that this lesion had a granular surface (Figure 2B). Pathological characteristics consistent with gastric MALT lymphoma were detected in biopsy specimens. Atypical lymphoid cells were found to have invaded the epithelium (lymphoep-

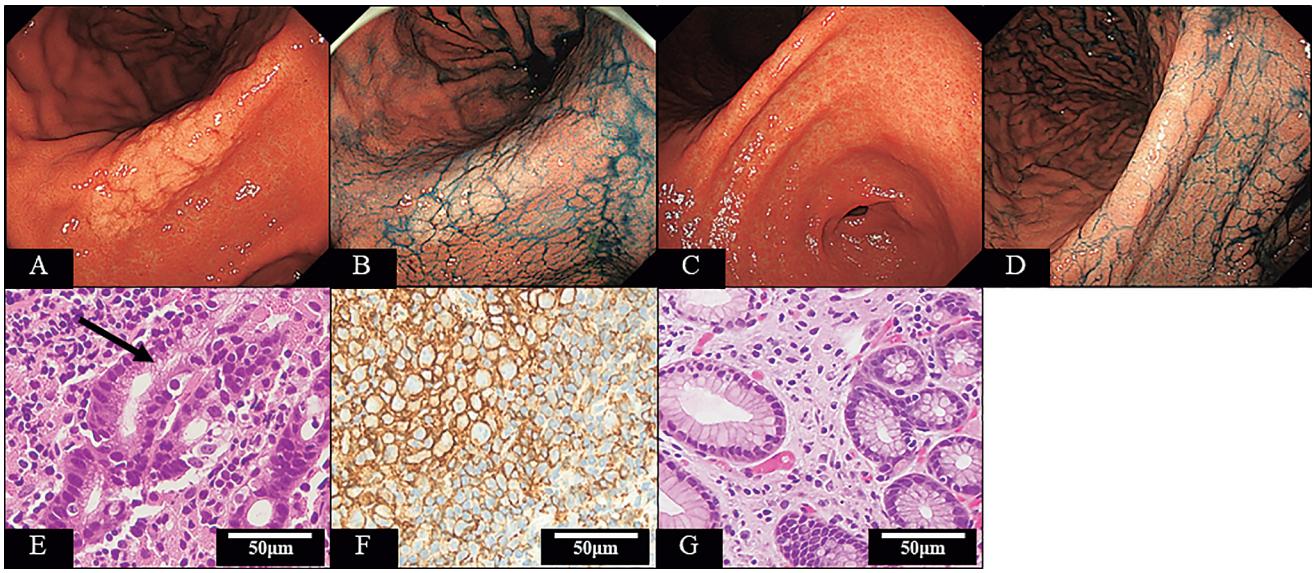

Figure 2 Endoscopic and histological observations before (A, B, E, F) and after (C, D, G) eradication therapy. Irregular protruding lesions on the angular incisure were observed by white light endoscopy (A) and indigo carmine chromoendoscopy (B). After eradication therapy, the surface irregularity of the gastric lesion improved. (C, D). Histological analysis by hematoxylin-eosin staining revealed a diffuse infiltrate of centrocyte-like cells and the formation of lymphoepithelial lesions (E). Immunohistochemical analysis showed that these centrocyte-like cells were positive for CD20 (F). Three months after eradication therapy, lymphoma cells had completely regressed (G).

ithelial lesions, Figure 2E). Immunohistochemistry assays revealed that CD20 staining was slightly greater than CD3 staining in each specimen (Figure 2F). Three months after the eradication of infection, the flat elevated lesion disappeared (Figure 2C, D) and CR was confirmed histopathologically (Figure 2G). In all three cases of *H. suis*-positive gastric MALT lymphoma, endoscopic and pathological observations were similar, and the efficacy of eradication therapy was confirmed using *H. suis* species-specific PCR. However, the lymphoma cells did not disappear after eradication therapy in one case (case number 7).

DISCUSSION

Numerous studies have reported the association between *H. pylori* infection and various gastric diseases. However, there are few reports regarding NHPH infections. *In vitro* cultivation of NHPH species is difficult and their urease activity is low[2,6]. Therefore, species-specific PCR analysis is considered to be the most accurate method of diagnosis in these cases[2,6].

In this study, we detected *H. suis* infection by PCR in *H. pylori*-negative gastric MALT lymphoma patients. *H. suis* was detected in 3 out of 15 cases (20%), using DNA extracted from background mucosa FFPE specimens. However, *H. suis*-specific bands were only detected in DNA isolated from FFPE tissues and not from FF samples. This discrepancy is not likely to be due to the fixation method, as DNA quality was confirmed by *GAPDH* amplification. However, it may be due to the location of the tissue sample and the number of biopsies taken. FFPE specimens were prepared to evaluate atrophic gastritis from various regions that contain normal gastric mucosa. The FF tissues were obtained through a pinpoint biopsy at the tumor site. Therefore, we hypothesize that *H. suis* may not colonize the lymphoma tumor site. Flahou et al. reported that *H. suis* infections spread throughout the stomach and are not localized to the lymph follicles[4]. Generally, *H. suis* tends to colonize the pyloric gland. Hence, when evaluating *H. suis* infection, biopsies from multiple sites, including the background gastric mucosa, should be used. The present study had several limitations that must be considered. Firstly, the number of patients was relatively small because we specifically selected *H. pylori*-negative patients. Secondly, the method of DNA extraction was different between biopsy specimens from tumor sites and normal mucosa. However, the PCR results reflected the effect of *H. suis* localization, rather than the quality of the extracted DNA.

Two of the three *H. suis*-positive lymphoma patients and 6 of the 12 *H. suis*-negative gastric MALT lymphoma patients showed CR after eradication therapy. The efficacy of eradication therapy was not significantly different between these two groups. It is possible that other NHPH species may also be involved in the pathogenesis of gastric MALT lymphoma. Therefore, additional studies using species-specific PCR analysis of a large number of patients are needed.

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