Association of host immunity with *Helicobacter pylori* infection in recurrent gastric cancer

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**Abstract**

**Background:** *Helicobacter pylori* infection is associated with the incidence of gastric cancer. Endoscopic resection has been developed as a proper technique to treat early stage of gastric cancer. However, some patients develop recurrent gastric cancer within 5 years after endoscopic treatment. The aim of the present study is to explore a biomarker for detecting people who has high risk of gastric cancer recurrence.

**Methods:** We analyzed the Interleukin-10 (IL-10) single nucleotide polymorphism (SNP) and IgG subclass responses to the bacteria in patients with early gastric cancer and recurrent gastric cancer.

**Results:** Patients with hetero-type in the 1082 SNP and CC genotype in the 592 SNP were at high risk of recurrence of gastric cancer. In patients with genotype carrying high risk of recurrence, IgG1 level tended to be higher than that in patients with other genotypes.

**Conclusions:** Dominance of T helper 2 (Th2) immunity controlled by IL-10 cytokine may be associated with *H. pylori*-associated gastric cancer recurrence.

**Keywords:** *Helicobacter pylori*, IL-10, Single nucleotide polymorphism, IgG subclass

**Background**

*Helicobacter pylori* infection is an important factor associated with gastric cancer [1]. Incidence of *H. pylori* induced gastric cancer is dependent on geographic factors, strain diversity, and host immunological responses [2]. The incidence of gastric cancer is higher in East Asian countries than other parts of the world [3, 4]. Therefore, several of the strain-specific features linked to high gastric cancer risk (including the cagA-ABD type of PAI, type s1 forms of vacA and babA) are present in nearly all East Asian *H. pylori* isolates [5–7]. However, the only minority patients were finally developed gastric cancer in the high carcinogenic *H. pylori* infected many patients. It is difficult to easily find a patient who develops gastric cancer among infected people.

Recently, endoscopic examination and resection has been established as a proper technique to treat early gastric cancer [8]. However, some patients developed recurrent gastric cancer within 5 years after endoscopic treatment. Previous studies reported that the incidence of recurrent gastric cancer within 5 years after endoscopic treatment is 3–10% [9, 10]. Clinicians recommend endoscopic examination every year, as it is difficult to distinguish patients with recurrent cancer. Therefore, identification of patients with high risk of recurrent cancer will be clinically and economically beneficial to the patients. We thought host immune response might be a potential marker to identify the patients with high risk of recurrent cancer.

*H. pylori* infection usually induces a strong T helper 1 (Th1) inflammatory response, which is characterized by cellular infiltration induced by cytokine and chemokine secretion. During the pathogenesis from chronic gastritis to gastric cancer caused by *H. pylori* infection, activated neutrophils and mononuclear cells in the host can produce pro-inflammatory cytokines, such as interleukin IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α,
and anti-inflammatory cytokines such as IL-10 [11, 12]. Therefore, host immunity is closely associated with gastric cancer risk, since gastric cancer usually develops in patients after more than 30 years of H. pylori infection.

In the context of anti-\(H.\, pylori\) immunity, studies on serum anti-\(H.\, pylori\) IgG subclass have indicated that subjects with low levels of IgG2 anti-\(H.\, pylori\) antibody were at a risk of gastric cancer [13, 14], suggesting that a decreasing Th1 response is associated with developing gastric cancer. IL-10 inhibits the production of pro-inflammatory cytokines by inhibition of Th1 lymphocytes, and stimulation of B lymphocytes and Th2 lymphocytes and thus downregulates the inflammatory response [15–17].

There are three frequently detected SNPs of IL-10 promoter gene, at position-1082 A/G and –819 C/T SNPs in its proximal promoter region and at position – 592 A/C SNP in its 5’-flanking region, which are associate with IL-10 production and development of gastric cancer [18–20]. The IL-10 promoter was also reported to be affected by the production of IL-10 [19–21].

In this study, we analyzed the IL-10 SNPs and IgG subclass responses to the bacteria in patients with early gastric cancer and recurrent gastric cancer.

Materials and methods
Patient information and DNA extraction
Samples from the patients were collected in Okayama University hospital with patients’ informed consent. Patients were treated by endoscopic mucosal resection (EMR) and followed up from 1 to 5 years. Patients’ information was shown in Table 1, and clinical character of recurrent gastric cancer patients was shown in Table 2. We obtained 10 to 20 paraffin sections of stomach tissue after first EMR of 49 cases of gastric cancer (20 cases of recurrence, 29 cases of recurrence within 5 years). DNA was extracted from these paraffin sections using PAX gene® Tissue DNA Kit and was stored at –80 °C.

PCR
The following primers were designed to amplify the IL-10 promoter region containing three SNPs for PCR: IL-10 forward primer (IL-10F) 5’-GTG GAA GGG GAA GGT GAA-3’ and IL-10 reverse primer (IL-10R) 5’-CCC AAG ACT TCT CCT TGC TA-3’. The cycling conditions consisted of an initial single cycle of a min at 98 °C, followed by 35 cycles of 10s at 98 °C, 30s at 60 °C, and 60s at 72 °C. Subsequently, the PCR product was diluted 50 times and a second PCR was performed using SNP-detection primers designed for the IL-10 region (Table 3). The cycling conditions when using primers of 819F, 819R, and 1082A-SNP consisted of an initial single cycle of a min at 98 °C, followed by 35 cycles of 10 s at 98 °C, 30 s at 66.5 °C, and 60 s at 72 °C. The annealing temperature was changed for the SNP detection primer without changing the other conditions; it was 66.5 °C for 1082G-SNP (same as 1082A-SNP), 57 °C for 819 T-SNP and 592A-SNP, 63 °C for 819C-SNP, and 60 °C for 592C-SNP. The obtained PCR product was electrophoresed on a 1% agarose gel.

Table 1 Patient characteristics

| Characteristic                  | Recurrent n = 20 | Not recurrent n = 29 | P value |
|--------------------------------|-----------------|----------------------|--------|
| Mean age, year (±SD)           | 72.1 (7.9)      | 65.2 (7.6)           | 0.002  |
| Gender (F/M)                   | 4/16            | 8/21                 | n. s.  |
| Observation period (month)     | 30 (33–118)     | 82 (26–99)           | 0.03   |
| Endoscopic atrophy (open/closed)| 19/3            | 20/9                 | n. s.  |

Table 2 Clinical character of recurrent gastric cancer patients

| Characteristic                  | Initial cancer | Recurrent cancer |
|--------------------------------|----------------|-----------------|
| Age, year (±SD)                 | 72.1 (7.9)     | 65.2 (7.6)      |
| Gender (F/M)                    | 4/16           | 8/21            |
| Observation period (month)      | 30 (33–118)    | 82 (26–99)      |
| Endoscopic atrophy (open/closed)| 19/3           | 20/9            |
| Macrophage classification       | Ilia           | Ilb              |
| Tissue type                     | tub1           | tub2             |

Table 3 Primer information

| Primer | Sequences of primers | Melting Temperature (°C) | Annealing Temperature (°C) |
|--------|-----------------------|--------------------------|----------------------------|
| IL-10F | 5’-GTG GAA GGG GAA GGT GAA-3’ | 55.8 | 60.0 |
| IL-10R | 5’-CCC AAG ACT TCT CCT TGC TA-3’ | 56.3 |     |
| 819F   | 5’-GAC TCC AGC CAC AGA AGC TTA C-3’ | 60.4 |     |
| 819R   | 5’-AGG TCT CTG GGC CTT AGT-3’ | 55.8 |     |
| 1082A-SNP | 5’-AAC ACT ACT AAG GCT TCT TGG TGA G-3’ | 57.3 | 66.5 |
| 1082G-SNP | 5’-AAC ACT ACT AAG GCT TCT TGG TGA G-3’ | 58.9 | 66.5 |
| 819T-SNP | 5’-TAC CCT TGT ACA GGT GAT GGA ATA-3’ | 57.1 | 57.0 |
| 819C-SNP | 5’-TAC CCT TGT ACA GGT GAT GGA ACA-3’ | 58.8 | 63.0 |
| 592A-SNP | 5’-TGA CCC CGC CGG TAC-3’ | 52.0 | 57.0 |
| 592C-SNP | 5’- TGA CCC CGC CGG TCC-3’ | 54.0 | 60.0 |

*Depend on the SNP detection primer
agarose gel at 100 V for 30 min, stained with ethidium bromide for 10 min, and then photographed.

ELISA
The levels of serum IgG subclass and total IgG antibodies against *H. pylori* antigen (cagA; ABD, vacA; s1c/m1, cagE+, iceA1, and babA2) were measured by ELISA. Ninety-six-well microtiter plates were coated with *H. pylori* lysate (50 μg/ml) in 100 μl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. After the wells were blocked with Phosphate Buffered Saline (PBS) containing 10% skim milk, the plates were incubated with sera containing 1:1000 of IgG and 1:100 of IgG1/2 for 2 h and washed with PBS containing 0.05% Tween 20. Peroxidase-labeled rabbit anti-human IgG1, IgG2, or IgG antibody (Dako Japan) was then added subsequently, and the plates were incubated for 2 h. After the plates were washed, 1 mg/ml of o-phenylenediamine (Wako Pure Chemical) in citrate buffer (pH 5.5) was added to the wells. The ODs were measured at 490 nm using an ELISA plate reader (Bio-Rad).

Statistical analysis
The recurrence/non-recurrence rate in each genotype was statistically analyzed using Fisher’s exact probability test. Welch's t-test was used for statistical analysis of each value of the IgG subclass. In both cases, *p* < 0.05 was considered significant, and a statistical software program (4 Steps Excel Statistics Ver.2, OMS, Japan) was used for all calculations.

Results
IL-10 polymorphisms with gastric cancer recurrence rate
The recurrence/non-recurrence rate of gastric cancer in the genotype for each SNP is shown in Fig. 1. The 1082 SNP was classified into homo genotype (AA, GG) and hetero genotype (AG); incidence of recurrent gastric cancer decreased in homo genotype (10% of AA and 28% of GG) of the 1082 SNP (Fig. 1a). A statistically significant difference was found between AA and AG genotypes in the 1082 SNP (*p* < 0.05). Eight hundred and-nineteen SNP was not associated with recurrent gastric cancer (Fig. 1b). Further, in the 592 SNP, a significantly (*p* < 0.05) high rate (86%) of CC genotype was associated with recurrent gastric cancer (Fig. 1c).

Association with allele/haplotype frequencies and recurrent/non-recurrent
Frequencies of ATA haplotype (1082A, 819 T, 592A) in non-recurrent gastric cancer were significantly higher than those in recurrent gastric cancer (Table 4). However, the GCC haplotype (1082G, 819C, 592C) was significantly increased in recurrent gastric cancer.

IgG antibody level in recurrent gastric cancer
The levels of total IgG and IgG subclass (IgG1 and IgG2) against *H. pylori* were measured (Fig. 2). IgG1 antibody was significantly higher in patients with recurrent cancer. To evaluate the diagnostic performance, ROC analysis was used. The ROC curves of IgG and IgG subclass are shown in Fig. 3. AUC values of total IgG, IgG1 and IgG2 were 0.58, 0.98, and 0.70, respectively.

| Frequencies (%) | ATA+ | GCC+ |
|-----------------|------|------|
| Recurrent (n = 20) | 11 (55%) | 12 (60%) |
| Non-recurrent (n = 29) | 22 (79%) | 11 (38%) |

*p = 0.0031 by chi-square test
Discussion
Gastric cancer remains one of the most common causes of cancer-related death. The incidence of gastric cancer prevails in a few cases among patients with H. pylori infection. Endoscopic resection has been established as a proper technique to treat early gastric cancer [19]. Some patients developed recurrent gastric cancer within 5 years after endoscopic treatment. Therefore, it is important to identify patients with high risk of recurrent gastric cancer. Some reports have indicated that IL-10 response and T helper polarity were associated with gastric cancer development. IL-10 strongly inhibits Th1 immunity, and Th1/Th2 balance affects producing IgG1/IgG2 balance. On the other hand, three SNPs of IL-10 promoter were effective to IL-10 production [21]. However, association with the SNPs and IgG1/IgG2 balance is obscure. No clear relationship was found between SNPs and IL-10 in this human study. Our study indicated that these host immunological feature and analysis of SNPs in the IL-10 promoter can be used to follow up after endoscopic resection of early gastric cancer. IgG1 (Th2 dominant antibody) response were available for prediction of recurrent gastric cancer.

Conclusion
The 1082 and 592 SNPs may be associated with gastric cancer recurrence. Patients with hetero-type in the 1082 SNP and CC genotype in the 592 SNP possess high risk of recurrence of gastric cancer. In these patients, systemic IgG1 level tended to be higher than other genotypes. Frequencies of ATA, GCC haplotype were associated with recurrent gastric cancer. Therefore, Th1/Th2 immunity balance controlled by IL-10 may be associated with the risk of H. pylori-associated gastric cancer recurrence.

Fig. 2 IgG antibody in patients with recurrent or not-recurrent gastric cancer. IgG and IgG2 antibody levels in patients with recurrent or not-recurrent were not different. IgG1 antibody level in patients with recurrent gastric cancer was significantly higher than not-recurrent patients.

Fig. 3 The ROC curves of IgG and IgG subclass in recurrent or non-recurrent gastric cancer. Total IgG (a), IgG1 (b), and IgG2 (c) to H. pylori in patients with recurrent or non-recurrent are shown. Cut off values of IgG, IgG1, and IgG2 are 0.29, 0.5, and 0.95, respectively.
Abbreviations
DNA: Deoxyribonucleic acid; ELISA: Enzyme-Linked ImmunoSorbent Assay; EMR: Endoscopic mucosal resection; Ig: Immunoglobulin; IL: Interleukin; PAI: Plasminogen activator inhibitor; PBS: Phosphate Buffered Saline; PCR: Polymerase chain reaction; SNP: Single nucleotide polymorphism; Th: T helper; TNF: Tumor necrosis factor

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
MS and CK performed main experiments, KM, HS, YO, and YK collected patients’ samples and information. OM, KY, and HO designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Ethical approval to carry out the study was obtained a priori from the Okayama Ethics approval and consent to participate University Ethics Committee (Number 2034). Written consent from the patient to participate this study was obtained.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Suebaun S, Michetti P. Helicobacter pylori infection. N Engl J Med. 2002;347:1175–86.
2. Cover TL. Helicobacter pylori diversity and gastric cancer risk. MBio. 2016;26:e01689–15.
3. de Maret C, Forman D, Plummer M. Gastric cancer: epidemiology and risk factors. Gastroenterol Clin North Am. 2013;42:219–40.
4. Yang L. Incidence and mortality of gastric cancer in China. World J Gastroenterol. 2006;12:17–20.
5. Van Doorn LJ, Figueiredo C, Mégraud F, Pena S, Midolo P, Queiroz DM, Carneiro F, Vanderborght B, Pegado MD, Sanna R, De Boer W, Schneeberger PM, Correia P, Ng EK, Atherton J, Blaser MJ, Quint WG. Geographic distribution of vacA allelic types of Helicobacter pylori. Gastroenterology. 1999;116:823–30.
6. Ito Y, Azuma T, Ito S, Miyai H, Hirai M, Yarnazaki Y, Sato F, Kato T, Kohli Y, Kuruya M. Analysis and typing of the vacA gene from caga-A-positive strains of Helicobacter pylori isolated in Japan. J Clin Microbiol. 1997;35:1710–4.
7. Lai CH, Kuo CH, Chen YC, Chao FY, Poon SK, Chang CS, Wang WC. High prevalence of caga-A and babA2-positive Helicobacter pylori clinical isolates in Taiwan. J Clin Microbiol. 2002;40:3860–2.
8. Kato M, Asaka M. Recent knowledge of the relationship between helicobacter pylori and gastric cancer and recent progress of gastroenteroscopic diagnosis and treatment for gastric cancer. Jpn J Clin Oncol. 2010;40:828–37.
9. Park JC, Lee SK, Seo JH, Kim YJ, Chung H, Shin SK, Lee YC. Predictive factors for local recurrence after endoscopic resection for early gastric cancer: long-term clinical outcome in a single-center experience. Surg Endosc. 2010;24:2842–5.
10. Hahn KY, Park CH, Lee YK, Chung H, Park JC, Shin SK, Lee YC, Kim HI, Cheong JH, Hyung WJ, Noh SH, Lee SK. Comparative study between endoscopic submucosal dissection and surgery in patients with early gastric cancer. Surgical Endoscopy Online ISSN. 2017;1432-2218:1–14.
11. Figueiredo CA, Marques CR, Costa Rosas S, da Silva HB, Alcantara-Neves NM. Cytokines, cytokine gene polymorphisms and Helicobacter pylori infection: friend or foe? World J Gastroenterol. 2004;20:5235–43.
12. El-Omar EM, Rabkin CS, Gammon MD, Vaughan TL, Risch HR, Schoenberg JB, Stanford JL, Mayne ST, Goedert J, Blot WJ, Fraumeni Jr JF, Chow WH. Increased risk of noncardia gastric Cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterol. 2003;124:1193–201.
13. Ren Z, Borody T, Pang G, Li LC, Dunkley M, Clancy R. Selective reduction of anti-helicobacter pylori IgG subclass antibody in gastric carcinoma. J Gastroenterol Hepatol. 2005;20:1338–43.
14. Martinez-Beccara F, Castillo-Rojas G, Ponce de Leon S, Lopez-Vidal Y. IgG subclasses against Helicobacter pylori isolates: An important tool for disease characterization. Scand J Immunol. 2012;76:26–32.
15. Mocellin S, Marincola FM, Young HA. Interleukin-10 and the immune response against cancer: a counterpoint. J Leukoc Biol. 2005;78:1043–51.
16. Avradopoulos K, Mehta S, Blackinton D, Wanebo HJ. Interleukin-10 as a possible mediator of immunosuppressive effect in patients with squamous cell carcinoma of the head and neck. Ann Surg Oncol. 1997;4:184–90.
17. Perrin GQ, Johnson HM, Subramaniam PS. Mechanism of interleukin-10 inhibition of T-helper cell activation by superantigen at the level of the cell cycle. Blood. 1999;93:208–16.
18. Ni P, Xu H, Xue H, Lin B, Lu Y. A meta-analysis of Interleukin-10-1082 promoter polymorphism associated with gastric Cancer risk. DNA Cell Biol. 2012;31:582–91.
19. Xue H, Lin B, An J, Zhu Y, Huang G. Interleukin-10 promoter polymorphism in association with gastric cancer risk. BMC Cancer. 2012;12:102–12.
20. Xue H, Wang Y-C, Lin B, An J, Chen L, Chen J, Fang J-Y. A meta-analysis of Interleukin-10-592 promoter polymorphism associated with gastric Cancer risk. Pros One. 2012;7:e93968.
21. Rad R, Dossurnbeke A, Neu B, Rauer S, Saur D, Gerhard M, Prinz C. Cytokine gene polymorphisms influence mucosal cytokine expression, gastric inflammation, and host specific colonization during Helicobacter pylori infection. Gut. 2004;53:1082–9.