Original Article

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Genetic Polymorphisms of Molecules Associated with Innate Immune Responses, TRL2 and MBL2 Genes in Japanese Subjects with Functional Dyspepsia

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Summary **Inflammatory changes in the gastric mucosa are commonly observed in Japanese patients with functional dyspepsia (FD). However, detailed data regarding the possible association between the genetic factors of inflammation related molecules and FD are not available. Toll like receptor 2 (TLR2) and mannan-binding lectin (MBL) protein play important roles in the innate immune activation. We aimed to clarify the association between common polymorphisms of TLR2 and MBL2 genes with FD in Japanese subjects. TLR2 −196 to −174 del and MBL2 codon54 G/A polymorphisms were genotyped in 111 FD patients according to Rome III criteria and 106 asymptomatic controls. Non-significant correlation was found between TLR2 and MBL2 polymorphisms with FD. However, in *Helicobacter pylori* (*H. pylori*) positives, we found significant inverse association between TLR2 −196 to −174 del carrier and FD among *H. pylori* positive subjects (Adjusted odds ratio (OR) = 0.48, 95% confidence interval (CI) = 0.23–0.996, *p* = 0.0488). We also found significant inverse association between the same genotype with postprandial distress syndrome (PDS) among *H. pylori* positive subjects (Adjusted OR = 0.22, 95% CI = 0.07–0.69, *p* = 0.0099). Our data suggest that TLR2 −196 to −174 del carriers's status but not MBL2 codon54 G/A is inversely related to the risk with FD in *H. pylori*-infected subjects.**

Key Words: **functional dyspepsia, TLR2, MBL2, *H. pylori*, polymorphism**

Introduction

Functional dyspepsia (FD) is a common clinical syndrome characterized by the presence of recurrent or chronic upper abdominal symptoms, such as epigastric pain, early satiety, and fullness, without anatomical or biochemical abnormality identifiable by conventional diagnostic tests, including upper gastrointestinal endoscopy [1]. Talley *et al.* [2] have shown that up to 25% of the population experienced these symptoms. FD is a heterogeneous condition indicated by the variety of different pathophysiologic mechanisms that have been demonstrated in this disorder [3], so FD does not have a well pathophysiology. Gastrointestinal motor abnormalities [4], altered visceral sensation [5] and psychosocial factors [6] have thought to be essential in the pathophysiology of FD. Recently, Locke *et al.* [7] reported familial clustering of FD. In addition, it has been reported that G-protein beta3 subunit gene polymorphism was associated with FD [8, 9]. These facts suggest that the genetic factor may play a significant role in the development of FD.

Helicobacter pylori (*H. pylori*) infection is a powerful pathogenic factor and many studies have revealed a strong

association between this organism infection and gastric disorders. *H. pylori* infection usually leads to persistent colonization and chronic gastric inflammation. According to the Rome III criteria [*10*], *H. pylori*-infected patients, who had some chronic or recurrent upper abdominal symptoms, with neither ulceration nor erosion in gastroduodenal mucosa by gastrointestinal endoscopy were diagnosed as FD. This indicates that one of the FD subgroups may relate to the gastric mucosal inflammation, although adult FD patients frequently have motility abnormalities of the stomach and upper small bowel including antral hypo-motility and delayed gastric emptying [*11–13*].

Cells of the innate immune system sense and respond to microbial products via the Toll-like receptors (TLRs). These receptors recognize conserved molecular patterns that are expressed by infectious agents. By this way, TLRs mediate the activation of transcription factors, mainly nuclear factor-kappa B (NF-κB) and proinflammatory cytokines resulting in inflammation [*14–18*]. Among the TLRs, TLR2 has been reported to be expressed in *H. pylori* infected gastric epithelial cells [*19*]. It has been also reported that *H. pylori* induced NF-κB activation and chemokine expression by gastric epithelial cells through TLR2. HEK293 cells that were stably transfected TLR2 resulted in extremely enhanced expression of interleukin 8 (IL-8), MIP-3α and GROα [*20*].

Mannan-binding lectin (MBL) protein, coded by the MBL2 human gene, is also an important constituent of the innate immune system. MBL initiates complement system but also function as an opsonin [*21, 22*]. Research over the past decade indicates that MBL provides a distinct third pathway of complement activation, which is called "the lectin pathway" and phylogenetic studies suggest that it may have been the first such pathway to have evolved [*23–25*]. It was reported that mucosal expression of MBL was up-regulated in *H. pylori* gastritis [*26*]. Furthermore, two recent studies of possible association of MBL2 haplotype and the susceptibility of *H. pylori* infection as well as the risk of gastric cancer were reported [*27, 28*].

Recently we have shown that common polymorphisms of TLR2 −196 to −174 del and MBL2 codon54 G/A (G54D) are associated with susceptibility to gastric cancer [*29, 30*], and the histological severity of *H. pylori* induced chronic gastritis [*31, 32*]. We have also shown that MBL2 codon54 G/A (G54D) are also associated with certain phenotypes of ulcerative colitis [*33*].

Since TLR2 and MBL may play a significant role in innate immune responses against *H. pylori* infection. We hypothesized that the polymorphisms of TLR2 and MBL2 gene may affect the severity of gastric mucosal inflammation by altering immune response against *H. pylori*, and modify the susceptibility to FD.

In the present study, we investigated the prevalence of TLR2 and MBL2 polymorphisms in patients with FD diagnosed according to the Roma III criteria in a Japanese population. We also wished to asses it's effect on the different subtypes of FD.

Materials and Methods

Study populations

Study populations were recruited from the subjects attending the Endoscopy Center of Fujita Health University Hospital from January 2006 to May 2008. This cohort is partly recruited from recent study [*34*].

So this was described in the study population. All the subjects underwent upper gastroscopy for their health check, secondary complete check up of stomach cancer following to barium X ray examination, or for the complaint of abdominal discomfort. Subjects who have significant upper gastrointestinal findings such as peptic ulcer disease, reflex esophagitis and malignancies were excluded from this study. Patients with malignancies in other organs, and had received non-steroidal anti-inflammatory drugs, antibiotics, and *H. pylori* eradication treatment were also excluded. Other diseases were also excluded by face-to-face history and physical examination including blood test, abdominal US and ECG. According to the Roma III criteria, 111 dyspeptic patients were identified as having a primary compliant of either continuous or intermittent dyspepsia for 3 months, onset at least 6 months before, predominantly located in upper abdomen irrespective of using H2-receptor antago-nists (H2RAS) or proton-pump inhibitors (PPIS). In 111 dyspeptic patients, 55 and 36 patients were diagnosed as epigastric pain syndrome (EPS) and postprandial distress syndrome (PDS) respectively.

Control subjects were recruited from the subjects who were negative for significant upper gastrointestinal findings, negative for dyspeptic symptom with in last 12 months and negative for abnormal finding for history and physical examinations. Finally, 106 asymptomatic subjects were included as non-dyspeptic healthy controls. Their reason for performing upper gastroscopy were, either for their health check or, for secondary complete check up of stomach cancer following to barium X ray examination. Those who had received or proton-pump inhibitory drugs or H2RAS during the 4 week were excluded from healthy controls. The Ethics Committee of Fujita Health University School of Medicine approved the protocol and written informed consent was obtained from all of the subjects.

Detection of H. pylori infection

H. pylori infection was determined on the basis of rapid urease test (RUT), serum antibody against *H. pylori*, and histological assessment using endoscopic biopsy specimens. During histological assessment, biopsy specimens were

obtained from non-pathological mucosa of greater curvature of the gastric antrum as well as upper corpus to avoid false-negative of *H. pylori* infection as much as possible. Infection was diagnosed when at least one of these 3 tests was positive.

Genotyping for TLR2 and MBL genes

Genomic DNA was extracted from non-neoplastic gastric biopsies or peripheral blood using the standard phenol/choloroform method. Polymorphisms at −196 to −174 del of TLR2 were investigated by allele specific polymerase chain reaction (PCR) method [29, 31]. PCR was performed in a reaction volume of 25 μl containing 200 ng of genomic DNA, 10 pmol of each primer, 200 ng of each dNTP and 0.6 units Taq DNA polymerase (Toyobo, Osaka, Japan). The primers for TLR2 were as follows: forward 5'-cacggag-gcagcgagaaa and reverse 5'-ctgggccgtgcaaagaag. The DNA was denatured at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 40 s. The final extension step was prolonged to 7 min. PCR products were visualized by electrophoresis on a 3.5% agarose gel by staining with ethidium bromide. One single band at 286 bp was judged as wild type (ins/ins), and a single 264 bp band was judged as homozygous type (del/del), while heterozygous type (ins/del) revealed two bands of 286 bp and 264 bp.

Polymorphisms at codon 54 G/A of MBL2 were investigated by restriction enzyme digestion with Ban I of amplified genomic DNA. PCR was performed in the same reaction volume as TLR2. The primers for MBL were as follows: forward 5'-ccttccctgagttttcacac-3' and reverse 5'-atcagtctcct-catatcccc-3'. The DNA was denatured at 95°C for 5 min, followed by 38 cycles of 95°C for 40 s, 55°C for 30 s, and 72°C for 40 s. The final extension step was prolonged to 7 min. The 298 bp PCR product (10 μl) was cleaved by *Ban I* (New England Biolabs. Inc, Bevely, MA) in an appropriate buffer at 37°C for 2 h.

PCR products were visualized by electrophoresis on a 3.0% agarose gel by staining with ethidium bromide. One single band at 298 bp was judged as homozygous type (54AA), and two bands of 195 bp and 103 bp was judged as wild type (54GG), while heterozygous type (54GA) revealed all three bands of 298 bp, 195 bp and 103 bp.

Statistical analysis

Hardy-Weinberg equilibrium of the TLR2 and MBL2 gene alleles in the control subjects were assessed by χ^2 statistics. Differences of genotype frequencies among two groups were determined by the two-sided Fisher's exact test. The odds ratio (OR) and 95% confidence interval (CI) were also calculated. A probability value of less than 0.05 was considered statistically significant in all analyses.

Results

Study population

A total of 111 FD patients including 55 EPS, 36 PDS, 26 other FD and 106 non-symptomatic control subjects participated in this study. The characteristics of the subjects are summarized in Table 1. Age distribution was not significantly different among those groups but female sex ratio was significantly higher in patients with over all FD, EPS and other FD than those of non-symptomatic control subjects. Female sex ratio tended to be higher in PDS than those of control. *H. pylori* infection positive ratio also tended to be lower in EPS than those of control.

TLR2 and MBL genotypes

Polymorphisms at −196 to −174 del of TLR2 was genotyped for 110 FD and all control subjects, while polymorphisms at codon 54 G/A of MBL2 was genotyped for all FD and 105 control subjects. The genotype distributions of both genes in all subjects are shown in Table 2. The frequency of TLR2 and MBL2 polymorphisms in the control subjects did not deviate significantly from those expected under the Hardy–Weinberg equilibrium ($p = 0.91, 0.34$). In over all subjects, no significant differences were found between both TLR2 and MBL2 genotypes frequencies and FD. Non significant association was also found between both TLR2 and MBL2 genotypes and subtypes of FD

Table 1.   Characteristics of subjects

Variables (n)	Sex (male:female)$	Age (mean ± SD)	*H. pylori* infection positive ratio (%)#
Controls (106)	64/42	60.9 ± 13.2	60.4
FD (111)	45/66	59.5 ± 13.6	53.2
EPS (55)	22/33	57.6 ± 14.3	45.5
PDS (36)	16/20	60.1 ± 13.6	47.2
Others (27)	8/19	62.7 ± 11.8	66.7

$; Control vs FD; $p = 0.004$, Controls vs EPS; $p = 0.02$, Controls vs PDS; $p = 0.1$, Controls vs Other FD; $p = 0.006$.
#; Control vs EPS; $p = 0.07$, statistical siginificant was assesed by χ^2 test.
Note; Seven of FD patients were diagnosed as both EPS and PDS.

Table 2. TLR2 and MBL2 polymorphisms and risk of FD

Variables n (%)	TLR2 genotype/n (%)			OR (95% CI)p	OR (95% CI)p
	ins/ins	ins/del	del/del	del/del vs others	ins/ins vs del carriers
Controls (106)	42 (39.6)	49 (46.2)	15 (14.2)	Reference	Reference
Over all FD (110)	52 (47.3)	44 (40.0)	14 (12.7)	0.88 (0.40–1.94) 0.84	0.73 (0.43–1.26) 0.27
EPS (55)	22 (40.0)	26 (47.3)	7 (12.7)	0.88 (0.34–2.32) 1.00	0.98 (0.51–1.91) 1.00
PDS (36)	20 (55.6)	14 (38.9)	2 (5.5)	0.36 (0.08–1.64) 0.24	0.53 (0.24–1.13) 0.12
Others (26)	13 (50.0)	7 (26.9)	6 (23.1)	1.82 (0.63–5.27) 0.37	0.66 (0.28–1.55) 0.38
Variables n (%)	MBL2 genotype/n (%)			OR (95% CI)p	OR (95% CI)p
	GG	GA	AA	AA vs others	GG vs A carriers
Controls (105)	62 (59.1)	35 (33.3)	8 (7.6)	Reference	Reference
Over all FD (111)	66 (59.5)	37 (33.3)	8 (7.2)	0.95 (0.34–2.63) 1.00	0.98 (0.57–1.69) 1.00
EPS (55)	30 (54.6)	19 (34.5)	6 (10.9)	1.50 (0.49–4.56) 0.56	1.20 (0.62–2.32) 0.62
PDS (36)	24 (66.7)	12 (33.3)	0 (0)	ND	0.72 (0.33–1.60) 0.55
Others (27)	17 (63.0)	8 (29.6)	2 (7.4)	0.98 (0.20–4.91) 1.00	0.85 (0.35–2.03) 0.83

Note: del carriers, del/del+ ins/del. A carriers, AA + GA. ND, not done.
One FD could not genotype for TLR2, and one control could not genotype for MBL2.
Statistical analysis was performed by two-sided Fisher's exact test.

according to Rome III.

To further evaluate the effect of TLR2 and MBL2 polymorphisms on the susceptibility of FD, we investigated the prevalence of both TLR2 and MBL genotypes in *H. pylori* positive and negative subjects respectively (Table 3). In the comparison of genotype frequency, we found significant inverse association between TLR2 −196 to −174 del carrier with FD among *H. pylori* positive subjects (ins/ins vs del carriers; OR = 0.47, 95% CI = 0.23–0.98, $p = 0.047$). We also found significant inverse association between the same genotype with PDS among *H. pylori* positive subjects (ins/ins vs del carrier; OR = 0.22, 95% CI = 0.07–0.70, $p = 0.01$). In *H. pylori* negatives, such correlation was not observed between the same genotype with the risk of FD including its subtypes according to Rome III. Non significant association was found between MBL2 genotype with the susceptibility of FD in different *H. pylori* infection status.

Logistic regression analysis

As female sex ratio was significantly higher in the FD patients than those of control, logistic regression analysis with adjustment for sex was done in *H. pylori* positive cases. We found that the significant association of the TLR2 −196 to −174 del carrier with FD and PDS was remained after logistic regression analysis (FD: OR = 0.48, 95% CI = 0.23–0.996, $p = 0.0488$, PDS: OR = 0.22, 95% CI = 0.07–0.60, $p = 0.0099$; Table 3).

Discussion

Although the relationship between inflammation and clinical presentation and treatment response is not well established in FD, histological inflammation has been implicated in the generation of gastrointestinal pain or discomfort [35]. Because *H. pylori* infection is a mainly pathogenic factor for gastric inflammation, it is suggested that interindividual differences of immune responses against *H. pylori* may influence the severity of gastric inflammation under the *H. pylori* infection and thus may modify the dyspeptic symptoms [34, 36].

In the present study, we investigated the association of the TLR2 −196 to −174 del and MBL2 codon54 G/A polymorphisms with FD. Although we failed to detect direct association between polymorphisms of those two polymorphisms and susceptibility to FD in over all subjects, we found that TLR2 −196 to −174 del carriers were significantly associated with a lower risk for developing FD especially PDS in *H. pylori*-infected subjects by stratification analysis.

It has been reported that TLR2 was expressed in *H. pylori* infected gastric epithelial cells [19] and that *H. pylori* induced NF-κB activation and chemokine expression by gastric epithelial cells was enhanced through TLR2 [20]. Noguchi *et al.* reported that the −196 to −174 ins/ins and ins/del genotypes of TLR2 was associated with higher transcriptional activity than del/del genotype by the luciferase reporter assay [37]. Recently, we have shown that the −196 to −174 ins allele of TLR2 was associated with histological severity of *H. pylori* induced gastric mucosal atrophy and intestinal metaplasia especially in older subjects, suggesting that the TLR2 genotype may modify the severity of gastric mucosal inflammation in the long term

Table 3. TLR2 and MBL2 polymorphisms and risk of FD in different *H. pylori* infection status

Variables n (%)	TLR2 genotype/n (%)			OR (95% CI)p	OR (95% CI)p
	ins/ins	ins/del	del/del	del/del vs others	ins/ins vs del carriers
H. pylori(−)					
Controls (42)	20	18	4	Reference	Reference
Over all FD (51)	21	21	9	2.04 (0.58–7.15) 0.37	1.30 (0.57–2.96) 0.67
EPS (30)	12	12	6	2.38 (0.61–9.29) 0.30	1.36 (0.53–3.52) 0.63
PDS (19)	8	9	2	1.12 (0.19–6.70) 1.00	1.25 (0.42–3.73) 0.78
Others (8)	3	3	2	3.17 (0.4–21.24) 0.24	1.52 (0.32–7.17) 0.71
H. pylori(+)					
Controls (64)	22	31	11	Reference	Reference
Over all FD (59)	31	23	5	0.45 (0.15–1.37) 0.19	0.47 (0.23–0.98) 0.047[#]
EPS (25)	10	14	1	0.20 (0.02–1.64) 0.17	0.79 (0.30–2.04) 0.63
PDS (17)	12	5	0	ND	0.22 (0.07–0.70) 0.01[$]
Others (18)	10	4	4	1.38 (0.38–4.99) 0.73	0.42 (0.14–1.21) 0.17

Variables n (%)	MBL2 genotype/n (%)			OR (95% CI)p	OR (95% CI)p
	GG	GA	AA	AA vs others	GG vs A carriers
H. pylori(−)					
Controls (41)	21	15	5	Reference	Reference
Over all FD (52)	25	23	4	0.60 (0.15–2.39) 0.50	1.13 (0.50–2.57) 0.84
EPS (30)	12	15	3	0.80 (0.18–3.64) 1.00	1.58 (0.61–4.09) 0.47
PDS (19)	12	7	0	ND	0.61 (0.20–1.87) 0.42
Others (9)	5	3	1	0.90 (0.09–8.80) 1.00	0.84 (0.20–3.58) 1.00
H. pylori(+)					
Controls (64)	41	20	3	Reference	Reference
Over all FD (59)	41	14	4	1.48 (0.32–6.90) 0.71	0.78 (0.37–1.66) 0.57
EPS (25)	18	4	3	2.77 (0.52–14.77) 0.34	0.69 (0.25–1.91) 0.62
PDS (17)	12	5	0	ND	0.74 (0.23–2.37) 0.78
Others (18)	12	5	1	1.20 (0.12–12.25) 1.00	0.89 (0.30–2.69) 1.00

Note: del carriers, del/del+ ins/del. A carriers, AA + GA. ND, not done.
One FD could not genotype for TLR2, and one control could not genotype for MBL2.
Statistical analysis was performed by two-sided Fisher's exact test.
[#]; Sex adjusted OR (95% CI)p = 0.48 (0.23–0.996) 0.0488.
[$]; Sex adjusted OR (95% CI)p = 0.22 (0.07–0.60) 0.0099.

out come of *H. pylori* infection [31].

Although we did not investigate the effect of TLR2 polymorphism on TLR2 activity in human gastric epithelial cells, it is possible that the polymorphism might have altered the activity of TLR2 in the human gastric mucosa. Since TLR2 plays important roles with respect to immunity response against *H. pylori*, it may be reasonable for the −196 to −174 del allele, which is associated with lower transcriptional activity to be associated with the reduced susceptibility to FD in *H. pylori* infected subjects.

We have also shown that the same genotype is associated with reduced susceptibility to *H. pylori* positive PDS according to Rome III. Holtmann *et al.* have reported that *H. pylori* positive individuals are as likely to have symptoms of bloating and early satiety as symptoms likely to PDS

[38]. On the other hand, it has been also reported that the abdominal pain score and indigestion score of the Gastrointestinal Symptoms Rating Scale are correlated to histological gastric mucosal atrophy and significantly decreased after eradication therapy in *H. pylori*-infected patients with ulcer-like FD [39, 40].

Since our data showed that *H. pylori*-infected patients with TLR2 −196 to −174 del carriers held lower risk of developing dyspeptic symptoms, especially PDS according to Rome III, *H. pylori* eradication may be recommended to other genotypes (homozygous TLR2 −196 to −174 ins) initially for prevention or for treatment of dyspeptic symptoms.

However, the association between *H. pylori* positive FD and TLR2 genotype is obtained from small samples, and the

type 2 error could not be excluded. This is a major limitation of our study and whether the TLR2 genotype may associated with Rome III subtypes needs further evaluation.

MBL is also an important constituent of the innate immune system in *H. pylori* infection. Codon 54AA genotype and A allele of MBL2 gene, which is associated with lower plasma concentration of MBL protein [41] are associated with gastric cancer [30] and histological severity of *H. pylori* induced gastric mucosal atrophy [32].

However, we did not find any association between MBL2 genotypes and FD. MBL2 polymorphism may be associated with gastric carcinogenesis and premalignant condition but not with the dyspeptic symptoms.

In conclusion, we have shown that TLR2 −196 to −174 del carriers's status but not MBL2 codon54 G/A is inversely related to the susceptibility to FD especially PDS in *H. pylori*-infected subjects.

Our data suggest that polymorphisms modulating the innate immune activations are associated with the FD under the influence of *H. pylori* infection. Our data also indicates the possible role of gastric mucosal inflammation in the pathophysiology of some subgroup of FD. Our study show that further longitudinal genetic studies will be needed in a larger, and ethnically diverse population to resolve the impact of the genetic polymorphisms of molecules associated with innate immune responses in the susceptibility to FD.

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