The *1244 A>G polymorphism of MyD88 (rs7744) is closely associated with susceptibility to ulcerative colitis

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Abstract. Toll-like receptor activation initially recruits the myeloid differentiation primary response gene (88) (MyD88) protein. A polymorphism *1244 A>G (rs7744) in the 3'-untranslated region of MyD88 has been identified. In the present study, the association of this polymorphism with ulcerative colitis (UC) was investigated. The population studied comprised 922 individuals, including patients with UC (UC cases) and without (controls). Genotyping of rs7744 was performed by PCR single-strand conformation polymorphism and the rs7744 G allele frequencies in the controls and UC cases were 32.8 and 43.5%, respectively (P<0.0001). The results showed that the genotype frequency of the AA homozygote was significantly lower and that of the GG homozygote was significantly higher in the UC cases compared with those in the controls (P=0.0012 for both groups). The rs7744 minor allele variants were significantly associated with susceptibility to UC as indicated by dominant and recessive genetic models. The minor allele variants were associated with an increased risk for UC in the male individuals but not the female individuals. The rs7744 was also associated with a non-continuous phenotype of UC and steroid unused/independent UC. This minor allele homozygote was associated with the disease severity of UC, hospitalization and response to steroid treatment. The results of the present study provided evidence that MyD88 polymorphism rs7744 was significantly associated with the development of UC and that this polymorphism may be associated with the response to treatment therapies for UC.

Introduction

Ulcerative colitis (UC) is precipitated by a complex interaction of environmental, genetic and immunoregulatory factors (1,2). Owing to the westernization of lifestyle and dietary habits, the number of UC patients has increased considerably in some Asian countries, including Japan and China (3). UC has an impact on the colon and rectum and usually involves the innermost lining mucosa, appearing as continuous areas of inflammation, with no segments of normal mucosa (4). Although the pathogenesis of UC is only partially understood, previous studies have indicated the significant role of the innate immune response in the pathogenesis of UC (5,6). In addition, a number of genes are associated with UC itself (7-10).

The biology of the innate immunity receptors is crucial in the host response to the environment. Toll-like receptors (TLRs) play a significant role in the gut innate immunity and are involved in human inflammatory bowel diseases, including UC (11). TLR activation initially recruits the myeloid differentiation primary response gene (88) (MyD88) protein, a common adaptor protein that is fundamental in the innate immune response activation (12). MyD88 has an intermediate domain that is crucial in TLR signaling as it interacts with interleukin-1 receptor-associated kinase. It has been reported that a polymorphism in the 3'-untranslated region (UTR) of MyD88 (rs7744, *1244 A>G) is associated with susceptibility to Buerger disease in Japanese individuals (13). MyD88 is located at chromosome 3p22 and its length is ~4.5 kbp. There is no other polymorphism excluding rs7744, with P>0.01 for Hardy-Weinberg equilibrium and P>0.05 for the minor allele frequency, within 10 kbp around MyD88 and no polymorphism in LD with rs7744 is observed within 20 kbp in the haplotype map of Japanese individuals (HapMap-JPT). In addition, there are binding sites of several microRNAs near *1244 A>G, including miR-29b-2-5p, miR-150-3p and miR-1236. Polymorphisms near the miRNA binding site in mRNA 3’-UTR may affect the binding of miRNAs to mRNA (14). Based on these results, we hypothesized that rs7744 has an impact on the MyD88-dependent pathway of the innate immune response via TLRs. It has been indicated that the TLR-4-MyD88 pathway may play a significant role on the pathogenesis of UC (15).

In the present study, the association of the MyD88 polymorphism rs7744 (*1244 A>G) with its susceptibility to UC was investigated.
### Materials and methods

**Clinical samples.** The studied population comprised 922 subjects, including patients with UC (UC cases, n=200), who were enrolled at the Fujita Health University Hospital or Kanazawa Medical University Hospital (Kutsukake-cho or Uchinada-machi, Japan), and subjects without UC (controls, n=722). The diagnosis of UC was based on standard clinical, endoscopic, radiological and histological criteria (16). The control subjects had no lower abdominal symptoms, diarrhea or hematochezia. Genomic DNA was isolated from peripheral blood using a FlexiGene DNA kit (QIAGEN GmbH, Hilden, Germany).

The Ethics Committees of Fujita Health University and Kanazawa Medical University approved the protocol, and written informed consent was obtained from the participating subjects.

**Sample size.** First, we assessed the genotype of 200 UC cases. Based on the frequency of the rs7744 minor allele in the UC cases (43.5%) in the present study an assumption was made that a 20% decrease in the prevalence of an allelic frequency would be of clinical relevance. Assuming $\alpha=0.05$ and power=0.80, at least 200 UC cases and 400 controls would be sufficient to identify a clinically relevant difference. Accordingly, 750 subjects without UC would be of sufficient clinical relevance for the study. A total of 722 of 750 subjects whose genotype was clearly determined were included as controls.

**Classification.** According to their clinical courses, the UC cases were classified into continuous and non-continuous disease (relapsing and only one episode) (16). UC patients were also classified as total colitis or non-total colitis (left sided, distal colitis and proctitis) according to the location and extension of the inflammatory lesions judged by the endoscopic findings. The cases that need continuous intravenous or oral steroid therapy were identified as steroid-dependent, and those that had one onset over 6 months or 2 onsets within one year were defined as refractory cases.

**Genotyping of polymorphisms.** Polymorphism was genotyped by the PCR single-strand conformation polymorphism (SSCP) method as reported previously (17,18). The primers used to detect rs7744 A>G were: MyD88 forward, 5'-ccttttctctttgtcctacactcattg-3' and reverse, 5'-cagctctcttcctctctgtgcttc-3'. A PCR reaction was carried out in a volume of 20 μl containing 0.1 μg genomic DNA. The DNA was denatured at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, 52°C for 40 sec and 72°C for 45 sec, with a final extension at 72°C for 5 min. Thereafter, 2 μl PCR product was denatured with 10 μl formamide (Sigma-Aldrich Co., St. Louis, MO, USA) at 90°C for 5 min. SSCP was carried out at 18°C using a GenePhor DNA separation system with GeneGel Excel 12.5/24 (GE Healthcare Japan, Tokyo, Japan), after which the denatured single-strand DNA bands were detected using a DNA silver staining kit (Amersham Biosciences Corp.).

**Statistical analysis.** The data of the age was expressed as the mean ± SD. The mean ages between the two groups were compared by the Student’s t-test. Allelic and genotype frequencies were calculated by direct counting. The allele counts and distribution of genotype were compared between the cases and the controls by a 2x2 table using Fisher’s exact test. Furthermore, the strength of the association between allele frequencies and the disease was assessed by calculating the odds ratio (OR) and 95% confidence intervals (CIs) by logistic multivariate regression analysis. For all the analyses, P<0.05 was used to indicate a statistically significant difference.

### Results

**Characteristics of subjects and the frequencies of genotypes.** As shown in Fig. 1, single-stranded DNAs were clearly separated by SSCP. The allele frequency of MyD88 rs7744 in the controls was in Hardy-Weinberg equilibrium (P=0.31). The mean age in the controls was significantly higher compared with that in the UC cases (Table I). The genotype frequency of the rs7744 AA homozygote was significantly lower and that of the GG homozygote was significantly higher in the UC cases compared with the controls (P=0.0012 and 0.0012, respectively). The minor allele frequency of rs7744 was 32.8 and 43.5% in the controls and UC cases, respectively (P<0.0001).

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Table I. Characteristics of the subjects and allelic frequency.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>UC cases</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>722</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Mean age ± SD (age of onset)</td>
<td>54.5±16.4</td>
<td>40.0±13.6</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Male:female</td>
<td>424:298</td>
<td>113:87</td>
<td>NS</td>
</tr>
<tr>
<td>rs7744 A&gt;G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>320</td>
<td>63</td>
<td>P&lt;0.0012</td>
</tr>
<tr>
<td>AG</td>
<td>331</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>71</td>
<td>37</td>
<td>P&lt;0.0012</td>
</tr>
<tr>
<td>G allele frequency</td>
<td>32.8%</td>
<td>43.5%</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

P-value, controls vs. UC cases. NS, not significant; UC, ulcerative colitis.
In the recessive genetic model, treatment for prob
genotype = 0.0029), but was

In the present study, the association between polymorphism
and the risk

On the other hand, the rs7744 polymorphism was
appeared not to have a difference because of disease severity

Moreover, the rs7744 polymorphism was significantly

Association between the rs7744 and UC.

UC cases (200)

Female

Table II. Association between rs7744 and UC.

Table: Association between rs7744 and UC.

Association between the rs7744 and UC. The rs7744 minor
allele was significantly associated with an increased risk
for UC using the dominant and recessive genetic models
(OR, 1.64; 95% CIs, 1.15-2.34; P=0.0063 and OR, 1.87; 95%
CIs, 1.17-3.00 and P=0.0094, respectively, Table II). In the
analysis using the number of minor alleles as a co-variate, the
significant association of the rs7744 minor allele was also
observed (OR, 1.52; 95% CIs, 1.18-1.95 and P=0.0010).
This association was observed in the male subjects in the
same manner (dominant genetic model: OR, 1.90; 95% CIs,
1.19-3.04 and P=0.045, and the number of minor
individuals who presented at hospital for
steroid-dependent or - unused cases in the dominant and recessive genetic
models. On the other hand, the rs7744 GA + GG genotype
was associated with cases without hospitalization and cases
with a UCDAI score <8, whereas the GG homozygote was
associated with cases with hospitalization, with a UDCAI
score >9 and steroid-dependent cases. These results indicate
that the rs7744 minor allele variant may be associated with
the development of UC and the minor allele homozygote with
the severity of UC. In the present study, sample selection may
have affected the outcome as the controls included unhealthy
individuals who presented at hospital for treatment for problems
excluding diarrhea, bloody feces and lower abdominal
discomfort. Although a comparison of allele frequencies
between the UC cases and controls have an adequately
statistical power (1-β=0.971), the effect of type II error
cannot be excluded by relatively small sample sizes in other
comparisons. Another limitation of the present study was
that the mean age was different among the controls and UC
cases. Therefore, an analysis was performed using a logistic
multivariate regression analysis following adjustment for age.
However, it appears that this is not an obstacle to the analysis,
as UC develops at a relatively young age.

It is well known that a major inducer of the inflammation
response to Gram-negative bacteria is lipopolysaccharide,
derived from the outer envelopes of these microorganisms (19).
Lipopolysaccharide signaling is mainly mediated through the

Discussion

In the present study, the association between polymorphism
rs7744 (*1244 A>G) in the 3′-UTR of MyD88 and the risk for
development of UC was evaluated. The rs7744 minor
allele variant was significantly associated with an increased
risk for UC, in particular in the male subjects. In addition,
this polymorphism was associated with cases with an older
age of onset, non-continuous disease and steroid-independent
or - unused cases in the dominant and recessive genetic
models.

Association between the rs7744 and phenotypes of UC.

The rs7744 polymorphism was associated with UC cases
with onset after 31 years of age by all the genetic models
(Table III). In addition, this polymorphism was significantly
associated with non-continuous disease and non-total colitis
by all the genetic models. In the recessive genetic model,
the rs7744 polymorphism was associated with non-total
and total colitis. On the other hand, this association of rs7744
appeared not to have a difference because of disease severity
or hospitalization. Moreover, the rs7744 polymorphism
was significantly associated with steroid-dependent or - unused
cases in all the genetic models, whereas this polymorphism
was associated with steroid-dependent cases only in the
recessive genetic model.
cell surface TLRs, which have been shown to be extremely important to gut homeostasis during host-microbial interactions (20,21). TLR-4 plays a significant role in gut innate immunity, protection and is involved in human inflammatory bowel diseases (IBDs), including UC (22,23). Cantó et al reported that a marked increase of TNF-α response to TLR2 ligands correlated with a higher TLR2 expression in Crohn's disease and UC patients, indicating that an abnormal mechanism may provide an excess of inflammatory mediators during the active phases of IBDs (24). This TLR signaling acts through a downstream regulator, MyD88, which initiates a signal transduction cascade leading to the induction of NF-κB (12). Although TLRs signal transmit through the MyD88-dependent or -independent pathway, previous studies have indicated a significant role of the TLR-MyD88 pathway on IBD (15,25). Aoyagi et al have reported that mRNA levels of MyD88, TLR-4 and NF-κB p65 are significantly increased in colonic mucosa of UC patients (26). It has also been reported that the Myd88 protein level is increased in the colonic mucosa of non-treated UC and this increase is suppressed by azathioprine treatment (15). Therefore, MyD88 is considered one of the key molecules in the pathogenesis of UC and an over-expression of MyD88 may promote the development of UC.

MyD88 is located on chromosome 3p22, a position that is not included in UC susceptible loci (27). However, rs7744, which is the only polymorphism in MyD88, may be associated with the susceptibility or pathogenesis of UC, if the expression and function of MyD88 undergo certain effects by the polymorphisms in MyD88. To the best of our knowledge, there is only one study regarding the investigation of an association between MyD88 polymorphism and human disorders. Although Chen et al have reported that rs7744 is associated with Buerger disease in Japanese individuals (131 cases and 270 controls) (13), the function of rs7744 has not been identified. In addition, there is no evidence regarding the function of rs7744. However, there may be a possibility of overexpressing the MyD88 protein in the rs7744 minor allele variants, considering overexpression of mRNA and protein levels of MyD88 in UC patients (15,26). There are binding sites of miR-150-3p and miR-1236 of 100 bp around rs7744. miRNAs bind to the RNA-induced silencing complex, making it non-symmetrical which then binds to the target mRNA to regulate its expression (28). In the rs7744 minor allele variants, cleavage of mRNA or repression of protein synthesis by miRNAs may not be well-regulated.

In the present study, MyD88 rs7744 was associated with non-continuous phenotypes of UC and steroid unused/independent UC. In addition, this minor allele homozygote was associated with the disease severity of UC, hospitalization and response to steroid treatment. Potter et al have reported that

### Table III. Association between rs7744 and the phenotype of UC.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Genotype, n</th>
<th>AG + GG vs. AA OR (95% CI)</th>
<th>GG vs. AA + AG OR (95% CI)</th>
<th>No. of G allele OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA  AG  GG</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Controls (722)</td>
<td>320 331 71</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Age of onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤30 (92)</td>
<td>33 42 17</td>
<td>1.19 (0.717-1.98)</td>
<td>1.51 (0.766-2.96)</td>
<td>1.22 (0.852-1.74)</td>
</tr>
<tr>
<td>≥31 (93)</td>
<td>27 49 17</td>
<td>1.90 (1.18-3.05)</td>
<td>2.03 (1.13-3.66)</td>
<td>1.67 (1.21-2.31)</td>
</tr>
<tr>
<td>Clinical type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not continuous (111)</td>
<td>31 58 22</td>
<td>1.91 (1.21-3.02)</td>
<td>2.02 (1.15-3.54)</td>
<td>1.67 (1.23-2.28)</td>
</tr>
<tr>
<td>Continuous (82)</td>
<td>31 38 13</td>
<td>1.23 (0.754-2.00)</td>
<td>1.58 (0.807-3.11)</td>
<td>1.25 (0.881-1.79)</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-total colitis (108)</td>
<td>31 57 20</td>
<td>1.82 (1.15-2.88)</td>
<td>1.89 (1.06-3.38)</td>
<td>1.61 (1.17-2.20)</td>
</tr>
<tr>
<td>Total colitis (89)</td>
<td>29 43 17</td>
<td>1.55 (0.955-2.52)</td>
<td>1.92 (1.04-3.56)</td>
<td>1.49 (1.06-2.09)</td>
</tr>
<tr>
<td>Max. UCDAI score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤8 (105)</td>
<td>32 56 17</td>
<td>1.74 (1.11-2.74)</td>
<td>1.68 (0.927-3.06)</td>
<td>1.52 (1.11-2.09)</td>
</tr>
<tr>
<td>≥9 (88)</td>
<td>30 40 18</td>
<td>1.37 (0.838-2.25)</td>
<td>2.01 (1.07-3.75)</td>
<td>1.42 (1.01-2.01)</td>
</tr>
<tr>
<td>Hospitalization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (121)</td>
<td>35 67 19</td>
<td>1.87 (1.21-2.88)</td>
<td>1.63 (0.919-2.90)</td>
<td>1.57 (1.16-2.12)</td>
</tr>
<tr>
<td>More than once (69)</td>
<td>26 29 14</td>
<td>1.19 (0.696-2.03)</td>
<td>2.05 (1.04-4.04)</td>
<td>1.33 (0.912-1.95)</td>
</tr>
<tr>
<td>Response to treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid-dependent (42)</td>
<td>17 14 11</td>
<td>1.03 (0.532-1.98)</td>
<td>2.71 (1.25-5.85)</td>
<td>1.37 (0.864-2.18)</td>
</tr>
<tr>
<td>Steroid-refractory (56)</td>
<td>18 27 11</td>
<td>1.46 (0.796-2.67)</td>
<td>1.88 (0.886-3.97)</td>
<td>1.44 (0.948-2.19)</td>
</tr>
<tr>
<td>Steroid independent or unused (155)</td>
<td>41 70 24</td>
<td>1.76 (1.17-2.66)</td>
<td>1.85 (1.08-3.16)</td>
<td>1.57 (1.18-2.09)</td>
</tr>
</tbody>
</table>

By logistic regression analysis following adjustment for age and gender. P-value: 0.0081; 0.018; 0.0018; 0.0054; 0.014; 0.0012; 0.011; 0.031; 0.0035; 0.038; 0.020; 0.016; 0.0087; 0.029; 0.045; 0.0047; 0.0055; 0.039; 0.011; 0.0668; 0.024 and 0.0020.
rs7744 is nominally associated with a response to anti-TNF therapy for rheumatoid arthritis (29). This evidence indicates that rs7744 may be associated with a response to treatment for UC. The reason for this genotype being associated with specific phenotypes or the male cases of UC remains to be determined. UC is a multi-factorial disorder including genetic and environmental factors, and is considered a complex genetic disorder predicted to involve multiple genes of relatively low penetrance (30). In fact, Fisher et al have reported that several regions of the male-specific linkage were identified in the susceptibility to inflammatory bowel disease (31). It may not be surprising that the MyD88 polymorphism is more closely associated with the specific phenotypes of UC. Future studies should be conducted to clarify how the MyD88 polymorphism affects the susceptibility to UC.

In conclusion, the minor allele variant of rs7744, which is located in MyD88 3'UTR, is significantly associated with the susceptibility to UC, in particular in the Japanese male subjects. This polymorphism may also be associated with the response to treatment for UC.

References