Inflammatory bowel disease (IBD) comprises mainly ulcerative colitis (UC) and Crohn’s disease (CD) characterised by long term intestinal inflammation attributable to an exaggerated immune response. The aetiology is still unknown. Genetic susceptibility has been shown by epidemiological and genetic investigations. Recent genome-wide linkage studies and case-control association studies have shown several susceptibility regions for IBD and identified the CARD15 (NOD2), the first susceptibility gene for CD.20 Linkage studies and case-control association studies have shown several susceptibility regions for IBD and identified CARD15 (NOD2), the first susceptibility gene for CD. NOD2 is a cytosolic protein expressed in monocytes and the innate immunity.

Recently, base-pair changes in the genes for both CD14 and toll-like receptor 4 (TLR4) have been described in humans. The LPS induced signalling through TLR4 and coreceptors CD14 as well as LPS binding protein (LBP) leads to NF-κB activation and cytokine expression.21 Two members of the TLRs family have been identified as an important link between innate and adaptive mucosal immune responses. TLR2 and TLR4 are suggested to be the main mediator of innate immune responses to LPS in vitro and in vivo. It seems that TLR4 is a principal signal transducer for LPS and TLR2 is a signal transducer for Gram positive bacterial components such as peptidoglycan lipopeptides and zymosan and certain structural variants of LPS.22 Mutations in the TLR4 receptor at residues 299 and 399 have been shown to be associated with hyporesponsiveness to inhaled endotoxin or LPS in humans. One of these missense polymorphisms in the fourth exon of TLR4 Asp299Gly changed the extracellular domain of the receptor. The mutation of TLR4 in mice was also shown to abolish responses to endotoxin, and to be hypersusceptible to Gram negative bacterial infection and to be insensitive to the LPS.23 24 Cario et al25 showed that normal intestinal epithelial cells and mucosa constitutively expressed TLR3 and TLR5 and hardly expressed TLR2 and TLR4, but TLR4 was strongly upregulated in both UC and CD. The results suggested that TLR4 might have a function in protein level in IBD. The study from Japan mentioned that there is no relation between TLR4 Asp299Gly and UC in Japan. However, Japanese patients with UC and CD14 C-260T in 114 patients with UC and 160 healthy controls in the Chinese Han population. Moreover a comparison was made with 170 healthy Dutch white subjects. Results: No TLR4 Asp299Gly mutation was detected in any patients or healthy controls in the Chinese Han population, which was similar to Japanese subjects, but the mutation occurred in 10% of the Dutch white subjects. There were no significant differences of CD14 genotypes between healthy controls and the patients with UC.

**Abbreviations:** UC, ulcerative colitis; CD, Crohn’s disease; IBD, inflammatory bowel disease; TLR4, toll-like receptor 4; LBP, LPS binding protein.
allele. Two papers have reported on the association between IBD and the polymorphism CD14 C-260T, but one is associated with UC while the other is associated with CD. Moreover, Torok et al have reported that they did not find the association between the CD14 promoter polymorphism and UC. Different results might be interpreted by genetic heterogeneity of races.

These studies investigated the possible role of TLR4/CD14 genes in LPS binding and signalling. We hypothesised that polymorphisms of the TLR4 and the CD14 gene may be important factors for determining susceptibility to UC and may be predictive for the disease outcome. To evaluate whether these two polymorphisms contribute to the predisposition to UC, we detected TLR4 and CD14 gene polymorphisms in Chinese patients with UC.

METHODS

Subjects

One hundred and fourteen patients with UC from Zhongnan Hospital of Wuhan University and other central hospitals in Wuhan city were studied. Diagnosis of UC was made by conventional clinical, radiological, endoscopic, and histological criteria. The patients with UC were classified according to sex (male and female) and location (left sided colitis, proctitis, and extensive colitis). Ethnically matched 160 Chinese healthy controls and 170 Dutch white healthy controls in the Chinese subjects, which is similar to the results in Japanese. However, mutations TLR4 Asp299Gly occurred in 10% of the Dutch subjects.

DNA isolation

Genomic DNA was isolated from 5 ml EDTA anticoagulated venous blood by conventional proteinase K digestion and phenol/chloroform extraction method. The positive control DNA samples of TLR4 and CD14 genes were provided by the laboratory of immunogenetics of VUmc.

TLR4 Asp299Gly genotyping

The two primer sets were designed from the TLR4 coding sequence (GenBank accession no U88880) referenced to Okayama et al (F1: 5’-TTAGAATGAGGAAACTTGGAAGAAG-3’, R1: 5’-TTGTGACAAATTATAAGTGATTAAT-3’, F2: 5’-AGCATACTAGACGACCTCAGTG-3’, R2: 5’-GTTGCCATCGGAAATTATAAGAAAG-3’). The PCR conditions were as follows: an initial denaturation at 94°C for three minutes, followed by 38 cycles of denaturing at 94°C for 45 seconds, annealing at 51°C for 45 seconds, and extension at 72°C for 45 seconds. The final extension was continued at 72°C for five minutes and cooling to 4°C. The PCR was performed using a total 25 µl of the following mixture: 50 pmol of each primer, 2.5 µl of 10×buffer, 1.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTPs, 1 µl of template DNA, 1 µl of AmpliTaq DNA polymerase (MBI Fermentas), and 13.5 µl ddH2O. The amplification was carried out on a Perkin-Elmer thermal cycler. PCR products were analysed by electrophoresis in 2% agarose gel with ethidium bromide staining under ultraviolet transillumination.

To detect the mutation in the TLR4 fragments, the PCR based restriction fragment length polymorphism (RFLP) analysis was performed. The PCR products were digested by the restriction enzymes BsaBI and BstXI. The 139 bp PCR product was amplified by F1 and R1, and the 131 bp fragment was amplified by F2 and R2. When digested by BsaBI, the fragments (139 bp for allele G and 112 bp +27 bp for allele A respectively) were obtained. When digested by BstXI, the fragments (108 bp +23 bp for allele G and 131 bp for allele A) were obtained. The products were separated by electrophoresis in non-denaturing polyacrylamide gels containing 8% acrylamide-bisacrylamide (29:1), 0.5× TRIS-borate-EDTA (TBE), 10% ammonium persulphate, and TEMED at 150 V for 1.5 hours. The gels were then subjected to silver staining.

CD14 genotyping

Primers of CD14 P1 (5’-CCTGCAGAATCCTTCCTGTT-3’) and CD14 P2 (5’-TCACCTCCCCACCTCTCTT-3’) were used to amplify a 107 bp fragment of the CD14 promoter (GenBank accession no U00699). A 25 µl PCR amplification mixture containing 50 pmol of each primer, 2.5 µl of 10×buffer, 1.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTPs, 1 µl of template DNA, 1 µl of AmpliTaq DNA polymerase, and 17 µl ddH2O, was run in a Perkin-Elmer Thermal cycler. After an initial denaturation at 94°C for five minutes, 35 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for one minute followed. The final extension was continued at 72°C for seven minutes and kept in 4°C. PCR products were analysed by electrophoresis in 2% agarose gel with ethidium bromide staining under ultraviolet transillumination. The C allele of the polymorphism of CD14 position 260 contains the restriction site for Hae III, which is not present in the T allele. The PCR was restricted with Hae III and the resulting fragments (107 bp for the T allele and 83 bp and 24 bp for the C allele, respectively) were separated on non-denaturing polyacrylamide gels containing 8% acrylamide-bisacrylamide and then silver staining.

Statistics analysis

The data were analysed using SPSS software (version 11.0, SPSS, Chicago, IL). Genotypes and allele frequencies were calculated by direct counting. Significance of the association was determined by χ² test and Fisher’s exact test. Departures from Hardy-Weinberg equilibrium were tested by the χ² method. A value of p<0.05 was considered significant. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated according to Woolf’s method.

RESULTS

In our study, the functional mutation Asp299Gly of the TLR4 gene was not detected in any Chinese subject. All the PCR products were sensitive to BsaBI and resistant to BstXI, they were all wild-type homozygotes. Therefore, significant association was not seen in patients with UC and healthy controls in the Chinese subjects, which is similar to the results in Japanese. However, mutations TLR4 Asp299Gly occurred in 10% of the Dutch subjects.

The distributions of genotype and allele frequencies of CD14 C-260T in both the patients and the healthy controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Healthy controls (n = 160)</th>
<th>UC (n = 114)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>T/T</td>
<td>58 (36.3)</td>
<td>42 (36.8)</td>
</tr>
<tr>
<td>C/T</td>
<td>77 (48.1)</td>
<td>62 (54.4)</td>
</tr>
<tr>
<td>C/C</td>
<td>15 (9.6)</td>
<td>10 (8.8)</td>
</tr>
<tr>
<td>Allele frequency</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>T</td>
<td>60.3</td>
<td>64.0</td>
</tr>
<tr>
<td>C</td>
<td>39.7</td>
<td>36.0</td>
</tr>
</tbody>
</table>
were in Hardy-Weinberg equilibrium (table 1). Frequencies of C/C genotype in healthy controls and UC were 15.6% and 8.8%, respectively, and frequencies of C/T genotype were 48.1% and 54.4%, respectively. The distribution of genotypes of the CD14 C-260T was not statistically different among the healthy controls and the patients with UC in the Chinese (p = 0.2267). In table 2, no significant differences were found in the genotype and allele frequencies of the CD14-260T among subgroups of UC, which were stratified according to sex and location of the disease. Furthermore, CD14 C-260T genotypes differed significantly in Chinese from Japanese,29 and from Dutch populations28 (table 3).

### DISCUSSION

This study has shown that the G allele of TLR4 gene Asp299Gly was not present in the Chinese population tested. Therefore, we could not find any association between the TLR4 Asp299Gly mutated genotype and UC, similar to a study in Japanese29 As they genotyped 108 healthy volunteers and 50 patients with UC and did not detect any mutation of the TLR4 Asp299Gly. Comparing the genotype and allele frequencies of the TLR4 Asp299Gly polymorphism among Chinese, Japanese,29 and Dutch white subjects,28 we found significant differences between Asians and Dutch white subjects. In Chinese and Japanese, the mutation of the TLR4 Asp299Gly did not occur, while in Dutch white subjects, the combination of AG and GG genotypes occurred in 10%. Our study showed that the distribution of the TLR4 Asp299Gly polymorphism was significantly different between Asia and this western population.

CD14, the coreceptor of TLR4 for LPS, is present in two forms, mCD14 and sCD14.33 34 Membrane CD14 is expressed on the surface of monocytes and macrophages and activates through TLR4, while sCD14 facilitates the LPS binding to the cells that do not express mCD14. CD14 gene is located on the long arm of chromosome 5 (5q31–33) and consists of a single base substitution (C→T) at position −260 in the promoter of the CD14 gene. This polymorphism has been reported to influence the density of CD14 expression on monocytes for the activation of monocytes to secrete inflammatory cytokines by LPS.28 The T variant of the −260 polymorphism can promote the transcription of CD14 gene and cause higher expression of CD14 on monocytes, which may lead to an increased inflammatory response. Furthermore, subjects carrying the T allele have significantly higher soluble CD14 levels than carriers of the C allele. Therefore, the CD14 polymorphism may be a genetic factor responsible for individual differences in the expression of CD14 and inflammatory response to luminal bacterial infections.

Zariee et al22 found that intestinal lamina propria mononuclear cells (LPMCs) of patients with CD were spontaneously activated by LPS. Active intestinal inflammation in IBD patients has shown increased expression of CD14 in LPMCs.33 34 Therefore, activation of the CD14 pathway in LPMCs or intestinal epithelium may be a trigger in the inflammatory response leading to increased production of proinflammatory cytokines and mucosal damage. Two clinical studies have shown an association between polymorphism in the promoter of the CD14 gene and myocardial infarction.23 35 One Japanese research group confirmed that the C-260T polymorphism in the promoter of the CD14 monocyte receptor gene was associated with acute myocardial infarction rather than with coronary atherosclerosis.23 Eng et al32 found an association between Chlamydia pneumoniae infection and CD14 polymorphism in the Chinese population in Taiwan. Linkage between CD14 polymorphism and IBD has also been studied. In a German population, an association of CD with allele T and the T/T genotype of CD14 gene has been found significant, which may be interpreted by a changed immune response to LPS contributing to the genetic predisposition to CD but not to UC.24 However, it has been shown that the CD14 gene in 5q31–33 region had a significant linkage with UC in the Chinese population16 and in the Canadian population.28

Our study has shown that CD14 C-260T polymorphism was not associated with UC in the Chinese population. The frequency of the C/C genotype in healthy controls (15.6%) was higher than in UC (8.8%), the C/T genotype in UC (54.4%) was higher than in healthy controls (48.1%), but neither of them is statistically significant. We did not find differences of CD14 C-260T between subgroups of UC as shown in table 2. Furthermore, the distributions of CD14 C-260T genotypes were significantly different Chinese and Japanese,27 and between Chinese and Dutch white subjects28 (table 3), which demonstrated ethnic genetic differences. The study implicates that the polymorphism of CD14 C-260T does not confer a genetic predisposition to UC. The lack of association of CD14 gene with UC in the Chinese population may be explained by genetic heterogeneity, and possibly a type 2 statistical error.

In summary, both the polymorphism of CD14 C-260T and TLR4 Asp299Gly were not associated with UC in the Chinese population, but the distributions of these two gene polymorphisms have ethnic differences.

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### Table 2: Genotype and allele frequencies of CD14 C-260T classified by sex and location of ulcerative colitis in Chinese patients

<table>
<thead>
<tr>
<th>Genotype Frequency (%)</th>
<th>Allele Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C/T</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male [n = 64]</td>
<td>23 (35.9)</td>
</tr>
<tr>
<td>Female [n = 50]</td>
<td>19 (38.0)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Left sided colitis [n = 51]</td>
<td>20 (39.2)</td>
</tr>
<tr>
<td>Proctitis [n = 19]</td>
<td>10 (52.6)</td>
</tr>
<tr>
<td>Extensive colitis [n = 44]</td>
<td>19 (43.2)</td>
</tr>
</tbody>
</table>

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### Table 3: Genotypes of CD14 C-260T in Chinese, Japanese, and Dutch population

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T/T</td>
<td>58 (36.3)</td>
<td>134 (25.4)</td>
<td>40 (24)</td>
</tr>
<tr>
<td>T/C</td>
<td>77 (48.1)</td>
<td>278 (52.8)</td>
<td>82 (48)</td>
</tr>
<tr>
<td>C/C</td>
<td>25 (15.6)</td>
<td>115 (21.8)</td>
<td>48 (28)</td>
</tr>
</tbody>
</table>

*Chinese v Japanese: \( \chi^2 (3,2) = 7.965, p = 0.0186; \)  
*Chinese v Dutch: \( \chi^2 (3,2) = 10.416, p = 0.0055 \)
ACKNOWLEDGEMENTS

We thank Professor H S Luo and Dr Y J Ding of the People’s Hospital of Wuhan University, Professor X H Hou and K F Zhou of Union Hospital and Professor N Z Liu and Associate Professor Q Zhao of Tongji Hospital of Huazhong Science and Technology University for assistance with collecting DNA samples for IBD patients. We also thank Ms Jolein Pleijster for assistance with typing most of the Dutch control cohort both CD14 and TLR4 genes.

Conflicts of interest: none.

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Funding: the study was supported by grants from the State Natural Science Foundation of China (30370638) and Hubei provincial public health bureau (JX1A14).

Conflicts of interest: none.

Ethics approval: all the subjects gave informed consent and the study protocol was approved by the ethics committee of Wuhan University Medical School.

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Polymorphisms of CD14 gene and TLR4 gene are not associated with ulcerative colitis in Chinese patients

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doi: 10.1136/pgmj.2004.030825