Lack of association between TYK2 and STAT3 genes and Crohn’s disease in the Malaysian population

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ABSTRACT. This study aimed to investigate the potential association of TYK2 and STAT3 genes with the susceptibility to Crohn’s disease (CD) among Malaysians. DNA samples were obtained from 80 CD patients and 100 healthy controls. Polymerase chain reaction-restriction fragment length polymorphism methods were employed for genotyping, followed by statistical analysis. In our current study, none of the single nucleotide polymorphisms of either TYK2 or STAT3 was statistically associated with the susceptibility to CD in our local population (P > 0.05). In contrast, there was a statistically significant association between the G/G homozygotes of the STAT3 rs2293152 and the healthy control
group ($\chi^2 = 6.229$, $P < 0.05$). In conclusion, our study does not support the role of the \textit{TYK2} and \textit{STAT3} genes influencing CD susceptibility.

\textbf{Key words:} Crohn’s disease; \textit{TYK2}; \textit{STAT3}; SNPs; Malaysia

\section*{INTRODUCTION}

Crohn’s disease (CD) and ulcerative colitis are 2 major forms of inflammatory bowel diseases (IBD), which involve inflammation of both the colon and small intestine (Baumgart, 2008). CD is characterized by transmural inflammatory lesions that affect virtually any part of the gastrointestinal tract (Raelson et al., 2007). In the past few decades, CD was thought to be a “western” disease, with higher prevalence rates reported in developed countries, relative to those of Asia (Yang et al., 2001; Goh, 2007). However, recent epidemiological studies have revealed an increasing trend in CD incidence and prevalence rates in this geographical region (Yang et al., 2001; Leong et al., 2004; Ouyang et al., 2005).

Although the etiopathogenesis of this disease remains unknown, its occurrence is thought to be triggered by atypical interaction between the intestinal mucosal immune response and enteric bacteria. CD is believed to be a multifactorial disease in which both genetic and environmental factors underlie its occurrence and development (Hugot et al., 2001). Genetically, the pericentromeric region of human chromosome 16 has been strongly associated with CD, as the most prominent CD susceptibility gene; nucleotide-binding and oligomerization domain 2/caspase recruitment domain 15 (\textit{NOD2/CARD15}) is located within the region (Hugot et al., 2001; Ogura et al., 2001). The 3 main disease predisposing mutations (R702W, G908R and 3020insC) of the \textit{NOD2/CARD15} gene were found to be in linkage with the susceptibility to CD in Caucasian populations (Hugot et al., 2001; Ogura et al., 2001, Cavanaugh, 2001). However, these mutations are absent in the Malaysian population, with the exception of JW1, SNP5 in \textit{NOD2/CARD15}, \textit{DLG5} and \textit{IBD5}, which were previously reported to be associated with CD (Chua et al., 2009a, 2011a, 2012).

There have been a significant number of studies conducted on other possible “candidate” genes such as interleukin-23 (\textit{IL23}), interleukin-23 receptor (\textit{IL23R}), disks large homolog 5 (\textit{DLG5}) and Toll-like receptor 4 (\textit{TLR4}). IL-23 plays an important role in regulating mucosal host defense; thus, it is involved in the pathogenesis of CD (Uhlig et al., 2006; McGovern and Powrie, 2007). In this context, we proposed that the components of the IL-23/IL-23R signaling pathway, i.e., tyrosine kinase 2 (\textit{TYK2}) and signal transducer and activator of transcription 3 (\textit{STAT3}), could possibly be linked with the susceptibility of CD. The \textit{TYK2} gene is located on the \textit{p} arm of chromosome 19, while the \textit{STAT3} gene is located on the \textit{q} arm of chromosome 17 (Lindqvist et al., 2000; Sehgal et al., 2003). Hence, in the present study, 3 single nucleotide polymorphisms (SNPs) were screened, namely, the \textit{TYK2} rs280519, \textit{TYK2} rs2304256 and \textit{STAT3} rs2293152.

\section*{MATERIAL AND METHODS}

\textbf{Sample collection and genomic DNA extraction}

A total of 80 CD patients and 100 healthy controls were recruited for this study. The collection of blood samples was approved by the Ethics Review Board of University Malaya
Medical Centre in Kuala Lumpur, Malaysia (Ethical approval No. 472.55). In addition, informed consent was obtained from each of the volunteers. Subsequently, genomic DNA was isolated from the whole blood by using a conventional DNA extraction method as stated previously (Tan et al., 2010). The isolated DNA was used for genetic analysis.

**Genotyping**

In this study, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was employed in order to investigate the distribution of both STAT3 and TYK2 gene polymorphisms, as well as their association with CD susceptibility. The primers used to analyze the STAT3 rs2293152, TYK2 rs280519 and TYK2 rs2304256 SNPs are listed in Table 1 (Sato et al., 2009). The PCR cycling parameters for the 3 product amplifications were set according to PCR conditions described elsewhere (Puah et al., 2007; Chua et al., 2010; Ng et al., 2012). These selected primers were then tested via in silico PCR as described previously for the prediction of amplicons prior to the actual PCR screening (Teh et al., 2010a,b; Thong et al., 2011; Chua et al., 2011b). Subsequent to the PCR step, the amplified products were digested with appropriate restriction enzyme (Table 1).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer sequence (5'-3')</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>TYK2</em> rs280519</td>
<td>F: 5'-CCG CCA TGG TGA AAG TTA GC-3'</td>
<td><em>Hpy</em>99I</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ATT TGT GCA GGC CAA GCT GC-3'</td>
<td></td>
</tr>
<tr>
<td><em>TYK2</em> rs2304256</td>
<td>F: 5'-TCA CCA GGC ACT TGT TGT CC-3'</td>
<td><em>BsmI</em></td>
</tr>
<tr>
<td></td>
<td>R: 5'-CGG CTT CCA GCA TGT GTA TG-3'</td>
<td></td>
</tr>
<tr>
<td><em>STAT3</em> rs2293152</td>
<td>F: 5'-TCC CCT GTG ATT CAG ATC CC-3'</td>
<td><em>HpaII</em></td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAT TCC CAC ATC TCT GCT CC-3'</td>
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</table>

Table 1. Primers and restriction enzymes (REs) for the analysis of *TYK2* rs280519, *TYK2* rs2304256 and *STAT3* rs2293152.

**Data analysis**

Similar statistical analysis as reported previously has been carried in this study (Chua et al., 2009b). The distribution of genetic polymorphisms was obtained by calculating both allelic and genotypic frequencies. As for the association testing, statistical analysis was performed by using the chi-square ($\chi^2$) test and measuring the odds ratio (OR) values.

**RESULTS**

In the analysis of the *TYK2* rs280519 SNP, all 3 genotypes, i.e., homozygous A/A, homozygous G/G and heterozygous A/G, were found in our local population. The presence of each genotype was indicated by different restriction fragments observed after *Hpy*99I enzyme digestion (Figure 1). In our study, none of the *TYK2* rs280519 genotypes ($P > 0.05$) were significantly associated with either the CD patients or healthy controls (Table 2).

As for the analysis of the *TYK2* rs2304256 C/A polymorphisms (Figure 2), the comparison between the genotype frequency distribution in CD patients and healthy controls did not reveal any significant differences ($P > 0.05$; Table 2). In addition, we did not observe any A/A homozygotes in either the cohort of CD patients or the healthy control group (Table 2).
Figure 1. PCR-RFLP of TYK2 rs280519. Lane 1 = 50-bp DNA ladder; lane 2 = PCR product without restriction enzyme digestion; lane 3 = heterozygous A/G; lane 4 = homozygous A/A; lane 5 = homozygous G/G; lane 6 = DNA blank.

Table 2. Genotypic frequencies (N), $\chi^2$ (P) and odd ratio (OR) and 95% confidence interval (95%CI) values of the TYK2 rs280519, TYK2 rs2304256 and STAT3 rs2293152 polymorphisms in Malaysian Crohn’s disease (CD) patients and normal healthy controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CD patient (%) (N = 80)</th>
<th>Control (%) (N = 100)</th>
<th>$\chi^2$ (P)</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYK2 rs280519</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>13 (16.2)</td>
<td>23 (23.0)</td>
<td>1.266 (P &gt; 0.05)</td>
<td>0.650 (0.3054-1.3819)</td>
</tr>
<tr>
<td>G/G</td>
<td>26 (32.5)</td>
<td>29 (29.0)</td>
<td>0.257 (P &gt; 0.05)</td>
<td>1.179 (0.6236-2.2284)</td>
</tr>
<tr>
<td>A/G</td>
<td>41 (51.3)</td>
<td>48 (48.0)</td>
<td>0.188 (P &gt; 0.05)</td>
<td>1.139 (0.6324-2.0511)</td>
</tr>
<tr>
<td>TYK2 rs2304256</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>38 (47.5)</td>
<td>49 (49.0)</td>
<td>0.040 (P &gt; 0.05)</td>
<td>0.942 (0.5228-1.6962)</td>
</tr>
<tr>
<td>A/A</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C/A</td>
<td>42 (52.5)</td>
<td>51 (51.0)</td>
<td>0.040 (P &gt; 0.05)</td>
<td>1.062 (0.5895-1.9127)</td>
</tr>
<tr>
<td>STAT3 rs2293152</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>23 (28.8)</td>
<td>47 (47.0)</td>
<td>6.229 (P &gt; 0.05)</td>
<td>0.455 (0.2439-0.8487)</td>
</tr>
<tr>
<td>C/C</td>
<td>16 (20.0)</td>
<td>10 (10.0)</td>
<td>3.596 (P &gt; 0.05)</td>
<td>2.250 (0.9592-5.2780)</td>
</tr>
<tr>
<td>G/C</td>
<td>41 (51.2)</td>
<td>43 (43.0)</td>
<td>1.215 (P &gt; 0.05)</td>
<td>1.394 (0.7720-2.5158)</td>
</tr>
</tbody>
</table>

In contrast, the analysis of the STAT3 rs2293152 polymorphisms revealed more encouraging results compared to those of the TYK2 loci. All 3 genotypes were present in all of our samples, and exhibited different banding patterns (Figure 3). The homozygous G/G genotype was also significantly associated with the healthy control group ($\chi^2 = 6.229, P < 0.05$; Table 2). This finding might suggest a “protective effect” of this genotype on the development of CD.
Figure 2. PCR-RFLP of TYK2 rs2304256. Lane 1 = 50-bp DNA ladder; lane 2 = PCR product without restriction enzyme digestion; lane 3 = heterozygous C/A; lane 4 = homozygous C/C; lane 5 = DNA blank.

Figure 3. PCR-RFLP of STAT3 rs2293152. Lane 1 = 100-bp DNA ladder; lane 2 = PCR product without restriction enzyme digestion; lane 3 = heterozygous G/C; lane 4 = homozygous C/C; lane 5 = homozygous G/G; lane 6 = DNA blank.

DISCUSSION

With respect to the TYK2 rs280519 SNP G/A gene polymorphisms, statistical analysis showed that both the A and G alleles were not significantly associated with CD ($\chi^2 = 0.944, P >$
The G allele, however, exhibited a higher disease penetration (OR = 1.231, 95% confidence interval (95%CI) = 0.8094-1.8719) as compared with its A counterpart (OR = 0.812, 95%CI = 0.5342-1.2355). Perhaps the intronic positioning of this SNP allows it to be spliced out during transcription, thus resulting in no correlation between it and susceptibility to CD progression.

Similarly, the TYK2 rs2304256 C and A alleles also did not differ significantly between both cohorts of samples ($\chi^2 = 0.026, P > 0.05$). While the homozygous genotype was absent in our results, this was due to the low frequency of the minor A allele. Similar observations have also been reported in the normal sampling of subjects in various other studies, i.e., Spanish (0.269), Japanese (0.38 and 0.345), Caucasians (0.25), and Malaysians (0.255) (Cunninghame Graham et al., 2007; Sato et al., 2009; Suarez-Gestal et al., 2009; Kyogoku et al., 2009, respectively). TYK2 rs2304256 is essentially a missense polymorphism in exon 8; hence, it is possible that while we did not observe any association with CD in isolation, it could be present on a number of neutrally transmitted low-frequency haplotypes in relation to other TYK2 SNPs.

In terms of the STAT3 rs2293152 polymorphisms, the G allele was significantly increased in the control group ($\chi^2 = 7.545, P < 0.05$) as compared to the C allele. This correlated to its lower OR value of 0.548 and 95%CI of 0.3561-0.8433; hence, perhaps the G allele acts as more of a “protective” factor against CD in our local population. This was also evident in terms of the GG homozygous carriers (Table 2). While Lovato et al. (2003) have demonstrated that STAT3 gene polymorphisms are associated with CD when studying intestinal T cells from CD patients, we also cannot rule out this possibility, since our current study has only focused on a single particular SNP (Lovato et al., 2003). There are a number of other possible candidate SNPs within the STAT3 gene that can be studied, including rs4769793, rs744166, rs6503695, and rs957970.

Previous studies have demonstrated that the occurrence of IBD involves a complex interplay of both innate and adaptive immune responses (Cho, 2008). In this setting, the IL-23/IL-23R signaling pathway is associated with CD susceptibility. IL-23, IL-23R, IL-12 precursor (IL-12B), IL-12 receptor (IL-12R), the Janus kinase (JAK) families and the STAT families are components of this complex network. Thus, both TYK2, a component of the JAK families, and STAT3 may play a role in conferring susceptibility to CD (Abraham and Cho, 2009; Wang et al., 2009). In the IL-23/IL-23R signaling pathway, TYK2 is activated via various cytokine receptors, and subsequently induces the phosphorylation, homodimerization and nuclear translocation of STAT3. As a result, the production of IL-17, natural killer T cells, CD4+ T cells, and CD8+ T cells are produced. This signaling cascade plays a central role in first-line host defense via the differentiation of naive T cells into TH17 cells (Watford et al., 2004; Nakamura et al., 2008). In addition, TYK2 will also bind to the interferon-α receptor (IFN-αR), where it is phosphorylated and activated (Richter et al., 1998). The active TYK2 will then phosphorylate the IFN-αR to allow further binding of STAT3 and STAT5 (David, 2002).

In our current study, both the TYK2 rs280519 and rs2304256 SNPs were not statistically associated with CD susceptibility. Interestingly, numerous polymorphisms of the TYK2 gene have been identified and several case-control studies have been conducted to investigate their association with other diseases, including autoimmune diseases. The results thus far are conflicting, although this could be due to several study-associated limitations. A meta-analysis performed by Tao et al. (2011) that investigated the possible linkage between these SNPs and human autoimmune diseases, found that TYK2 rs2304256 was associated with autoimmune
diseases, while rs280519 was not. Similarly, there was no statistically significant association between STAT3 rs2293152 and CD susceptibility in our Malaysian population. In conclusion, our current study demonstrated that both the TYK2 and STAT3 genes were not found to be major risk factors related to CD susceptibility in our local population.

ACKNOWLEDGMENTS

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REFERENCES


