Original Article

Interleukin-13 genetic polymorphisms in Singapore Chinese children correlate with long-term outcome of minimal-change disease

Chang-Li Wei, Wai Cheung, Chew-Kiat Heng, Novi Arty, Samuel S. Chong, Bee-Wah Lee, Ken-Lee Puah and Hui-Kim Yap

Department of Pediatrics, National University of Singapore, Singapore

Abstract

Background. Minimal-change nephrotic syndrome (MCNS) has been associated with atopy. As interleukin-13 (IL-13) has been implicated in the pathogenesis of MCNS, we postulated that IL-13 genetic polymorphisms could influence either susceptibility or clinical course of the disease.

Methods. Seventy-two Singapore Chinese children with MCNS and 78 normal controls were screened for single nucleotide polymorphisms (SNPs) in the IL-13 gene by direct sequencing. Allele and genotype frequencies of these SNPs were determined and their relationship with different clinical courses was analysed.

Results. Six SNPs were identified in the 5′ promoter, exon 4 and 3′ untranslated region (3′UTR). The three SNPs in the 3′UTR – 4738 (G/A), 4793 (C/A) and 4926 (C/T) – were in tight linkage disequilibrium (Δ ≥ 0.99). There was no difference in allele or genotype frequencies between MCNS children and normal controls. However, there was a significantly lower frequency of allele 4738G in those MCNS children who were still relapsing after 5 years of follow-up (G = 0.52), compared with those in complete remission (G = 0.72; P < 0.05) and normal controls (G = 0.69; P < 0.05). Haplotype analysis showed a significantly higher frequency of the GCC haplotype in controls and MCNS patients in complete remission (χ² = 6.35; P < 0.02), while the frequency of AAT haplotype was higher in those MCNS children still relapsing after 5 years of follow-up (χ² = 5.38; P < 0.02). Moreover, peripheral blood mononuclear cell IL-13 mRNA expression in patients with haplotype AAT was significantly higher than in those with haplotype GCC.

Conclusions. These results suggest that genetic polymorphisms in the 3′UTR of the IL-13 gene correlate with long-term outcome of MCNS, rather than disease susceptibility, in Singapore Chinese children.

Keywords: genetic polymorphism; interleukin-13; minimal-change nephrotic syndrome; prognosis; Singapore Chinese children

Introduction

Minimal-change nephrotic syndrome (MCNS) is a glomerular disease that is characterized by heavy proteinuria and a relapsing/remitting course, without histological evidence of classical inflammatory immune-mediated injury. It is the most common cause of nephrotic syndrome in Singapore Chinese children. Although its pathogenesis remains to be elucidated, there is strong evidence that it is a primary immune disease associated with immunoregulatory imbalance between Th1 and Th2 cytokines [1].

The close relationship between atopy and MCNS suggests a common immune pathway. Stimuli, such as allergens, may activate common immune mechanisms, which subsequently result in proteinuria in children with MCNS. Several investigators have demonstrated the potential role of Th2 cytokines in children with MCNS. Several investigators have demonstrated the potential role of Th2 cytokines in this disease [2–4]. Previous studies suggested that both interleukin (IL)-4 and IL-13 independently enhance the production of IgE in non-atopic donors, while interferon-γ inhibits the event [5]. In MCNS, Cho and co-workers [6] have shown that increased peripheral blood mononuclear cell (PBMC) production of IL-4 was associated with enhanced expression of type II immunoglobulin (Ig)-E receptor expression on B cells and high IgE production. Our group has demonstrated that IL-13 gene expression was upregulated in both CD4+ and CD8+ T cells in children with steroid-responsive nephrotic syndrome in relapse, compared with remission [4]. This was associated with increased intracytoplasmic IL-13 production by

Correspondence and offprint requests to: Hui-Kim Yap, MD, Department of Pediatrics, National University of Singapore, 10 Lower Kent Ridge Crescent, 119074 Singapore. Email: paeyaphk@nus.edu.sg

© The Author [2005]. Published by Oxford University Press on behalf of ERA-EDTA. All rights reserved. For Permissions, please email: journals.permissions@oupjournals.org
CD3+ cells and elevated serum total IgE levels during relapses. Moreover, the elevated serum total IgE levels found in nephrotic patients in relapse correlated significantly with the increase in CD3+ IL-13-producing cells. These findings were consistent with Kimata et al.’s [2] study, where IL-13 was shown to be important for the spontaneous production of IgE and IgG4 by PBMCs in nephrotic patients, in contrast to atopic individuals where IL-4 was the controlling cytokine. Van den Berg et al. [7] also demonstrated the presence of IL-13 receptors on glomerular epithelial cells, and stimulation of these receptors resulted in a decrease in transepithelial electrical resistance, suggesting possible direct effects of this cytokine on podocytes.

IL-13 is an important immunoregulatory protein that is produced by different T-cell subsets and dendritic cells. It consists of 132 amino acids and has a molecular mass of 12 kDa. The human IL-13 gene is located on chromosome 5q31, in the same cluster of genes encoding IL-3, IL-4, IL-5, IL-9 and granulocyte–monocyte colony stimulating factor. In particular, IL-13 shares many biological activities with IL-4, which include IgE isotype switching, CD23 induction, stimulation of eosinophil activity, mucus production and smooth muscle activity in the airway. This is due to the fact that they share a common IL-4 receptor α-chain (IL4RA) in the multimeric IL-4 and IL-13 receptor complexes [8].

In recent years, IL-13 has come to be appreciated as a critical mediator in allergic disease, and numerous studies have shown that the genetic polymorphisms of IL-13 and its receptor were associated with elevated IgE levels, atopy and/or asthma in different populations [9–13]. However, earlier studies have failed to demonstrate any association between these polymorphisms and MCNS [14]. Hence, to further elucidate the relationship between IL-13, atopy and MCNS, we examined the IL-13 gene, including its 5' promoter and 3' untranslated region (3'UTR), for single nucleotide polymorphisms (SNPs) in Singapore Chinese children with MCNS and investigated their relationship with atopy, steroid-response and long-term outcome.

Subjects and methods

Subjects

Seventy-two unrelated children with MCNS (46 boys and 26 girls) were included in this study. Their mean age at diagnosis was 4.5±2.1 years (range: 1–12 years). Sixty-six children were steroid-sensitive, with features which fulfilled the clinical criteria of MCNS as defined by the International Study of Kidney Disease in Children [15], in that they were normotensive, had normal serum creatinine and complement levels, and had no gross haematuria during their clinical course. Six children were steroid-resistant, as defined by a failure to achieve remission despite 6 weeks of daily high-dose steroid therapy, and all of them had a renal biopsy which confirmed the presence of MCNS. Relapse was defined as increased urinary protein excretion (Albustix® ≥2+ for at least three consecutive days or >40 mg/m²/h) and serum albumin ≤25 g/l. Remission was defined as serum albumin ≥35 g/l and normal protein excretion (Albustix® trace or negative for at least three consecutive days or <5 mg/m²/h) [15]. Patients were defined as having frequent relapses if they had four or more relapses within any 1 year period, while steroid-dependency was defined as having consecutive relapses while on steroid therapy or within 2 weeks of cessation of steroid therapy. Thirty-eight of the patients were steroid-dependent, of whom 19 had a renal biopsy confirming MCNS, while 28 were infrequent relapsers. Clinical parameters of atopy, namely asthma, eczema, recurrent urticaria and allergic rhinitis, were present in 14 children. All the children with MCNS had completed ≥5 years of follow-up (mean: 8.3±4.0 years; range: 5–25 years), of whom 43 were in complete remission, whilst 29 were still relapsing. Additionally, 78 gender- and age-matched healthy Chinese children formed the control group (50 boys and 28 girls). Informed consent was obtained from the parents prior to blood sampling and this study was approved by the National University Hospital Institutional Review Board.

Genotyping

Genomic DNA from patients with MCNS and controls was screened for genetic variants of the IL-13 gene using a direct polymerase chain reaction (PCR)-sequencing method. Nucleotide numbering was based on the GenBank accession no. U31120 as the reference sequence. PCR products were generated by overlapping sets of primers designed to amplify all four exons and flanking intronic sequences, the 1785 bp promoter region and the 765 bp 3'UTR of the human IL-13 gene (Table 1). PCR amplifications were carried out in 25-μl volumes containing...
~40 ng genomic DNA, 20 pM upstream or downstream primers, 200 μM dNTP and 0.5 units Taq DNA polymerase. The amplification procedure consisted of 30–35 cycles with the following parameters: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 0.5 min on a GeneAmp 9700 system (PE Applied Biosystems, Foster, CA, USA). Before sequencing, the PCR products were purified with QIAquick PCR purification kit (QIAGENE, Hilden, Germany). ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (PE Applied Biosystems) were then used for double-strand sequencing of the PCR products. Sequencing was performed on a GeneAmp PCR 9700 system (PE Applied Biosystems). The sequenced products were purified by ethanol precipitation, following which the DNA pellet was dissolved in 4 μl of loading dye (deionized formamide, 50 mg/ml dextran, 25 mM EDTA, pH 8.0) and analysed on an Applied Biosystems Genetic Analyser 3100 (PE Applied Biosystems). The sequenced products were aligned to the reported human IL-13 gene (GenBank accession no. U31120), in order to screen for SNPs.

IL-13 mRNA expression

IL-13 mRNA expression in PBMCs from patients with the different IL-13 haplotypes was measured using reverse transcription (RT)–PCR. In brief, total RNA was extracted from PBMC(s) of MCNS patients with known haplotypes GCC or AAT, using TRIzol™ Reagent (Gibco BRL, Grand Island, NY, USA). The patients studied were in remission and off steroid treatment, in order to exclude the effects of prednisolone on IL-13 mRNA expression. Integrity of the total RNA was checked with formaldehyde agarose gel and indicated by the presence of the 28S and 18S rRNA bands. RT was performed with oligodT using SUPERSCRIPT II RNase H– reverse transcriptase (Gibco BRL, Karlsruhe, Germany), following the manufacturer’s protocol. PCR was done using a DNA amplification reagent kit (Qiagene) with GeneAmp PCR system 9700 (Applied Biosystems). The primers for IL-13 were 5’-ATTGCTCTCACTTGGCCTT (forward) and 5’-CTAACCCCTCCTCCCGCTA-3’ (reverse). The primers for the house-keeping gene cyclophilin A were 5’-GTGACTCACAAGCCATAATGCG-3’ (forward) and 5’-GGTGCTCTCTGAGCTACAGAA GG-3’ (reverse). It was used to standardize the expression of IL-13. PCR products were then resolved in agarose gel, electrophoresed and visualized by ethidium bromide staining. Results were presented as an index of the band intensity.

Statistical analysis

The chi-square test ($\chi^2$) was used to check for significant departure from Hardy–Weinberg expectation. The difference in the allele and genotype frequencies between groups was determined by chi-square or Fisher’s exact test. Linkage disequilibrium between different polymorphisms was assessed by delta ($\Delta$) [16]. The assignment of haplotype using genotype information was carried out using Arlequin v. 2.000 (Genetics and Biometry Laboratory, University of Geneva, Switzerland). Statistical significance was defined as $P<0.05$.

Results

Using the direct PCR-sequencing method with overlapping primers, we detected six known SNPs of the IL-13 gene in our population of Singapore Chinese children. As shown in Table 2, one SNP was detected in exon 4, two in the 5’ promoter region and three in the 3’UTR. The genotype frequencies of all these SNPs were in agreement with the Hardy–Weinberg equilibrium. In contrast, there were no SNPs detected within the coding regions of exons 1, 2 and 3.

In exon 4, a non-synonymous SNP was located at 4257 (G/A, R130Q). The major allele (G, Arg130) frequency was 0.65 in both MCNS patients as well as controls (Table 3). In the promoter region, the

<table>
<thead>
<tr>
<th>Locus</th>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Controls (%)</th>
<th>MCNS (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ Promoter</td>
<td>704</td>
<td>AA</td>
<td>51 (65.4)</td>
<td>42 (58.3)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC</td>
<td>23 (29.5)</td>
<td>28 (38.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>4 (5.1)</td>
<td>2 (2.8)</td>
<td></td>
</tr>
<tr>
<td>5’ Promoter</td>
<td>1103</td>
<td>CC</td>
<td>60 (76.9)</td>
<td>56 (77.8)</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>18 (23.1)</td>
<td>16 (22.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Exon 4</td>
<td>R130Q</td>
<td>GG</td>
<td>32 (41.0)</td>
<td>33 (45.8)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>38 (48.7)</td>
<td>27 (37.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>8 (10.3)</td>
<td>12 (16.7)</td>
<td></td>
</tr>
<tr>
<td>3’UTR</td>
<td>4738</td>
<td>GG</td>
<td>37 (47.4)</td>
<td>32 (44.4)</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>34 (43.6)</td>
<td>29 (40.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>7 (9.0)</td>
<td>11 (15.3)</td>
<td></td>
</tr>
<tr>
<td>3’UTR</td>
<td>4793</td>
<td>CC</td>
<td>37 (47.4)</td>
<td>32 (44.4)</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA</td>
<td>34 (43.6)</td>
<td>28 (38.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>7 (9.0)</td>
<td>12 (16.7)</td>
<td></td>
</tr>
<tr>
<td>3’UTR</td>
<td>4962</td>
<td>CC</td>
<td>37 (47.4)</td>
<td>32 (44.4)</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>34 (43.6)</td>
<td>29 (40.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>7 (9.0)</td>
<td>11 (15.3)</td>
<td></td>
</tr>
</tbody>
</table>
two SNPs detected were located at 704 (A/C) and 1103 (C/T), which occur at −1510 and −1111 upstream of the open reading frame, respectively. The A allele frequency of the SNP at 704 was 0.78 in children with MCNS as compared with 0.80 in non-atopic controls, while the C allele frequency of the SNP at 1103 was 0.89 in children with MCNS compared with 0.80 in non-atopic controls, while the C allele frequency of the SNP at 704 was 0.78 in children with MCNS compared with 0.80 in non-atopic controls.

We further stratified the children with MCNS according to the presence or absence of atopy, steroid-responsiveness, steroid-dependency and long-term remission after ≥5 years of follow-up and subsequently evaluated the potential association with IL-13 genetic polymorphisms. There was no significant difference in the allele or genotype frequencies for all the SNPs in the MCNS children stratified for atopy, steroid-responsiveness and steroid-dependency. However, the G allele frequency of the 3′UTR SNP 4738 was significantly reduced in the MCNS patients who were still relapsing after 5 years of follow-up, as compared with those in complete remission (χ² = 5.38; P < 0.05) (Table 5) as well as normal controls (χ² = 4.92; P < 0.05).

Haplotype analysis was performed subsequently for the three SNPs in the 3′UTR. Due to the tight linkage disequilibrium between the three 3′UTR SNPs, only three haplotypes GCC, AAT, GAC were demonstrated, although the mathematical probability of haplotypes was 8. The third haplotype (GAC) was rare, occurring in only one patient (1.7%). As shown in Table 6, the GCC haplotype was significantly higher in the MCNS patients who were still relapsing after 5 years of follow-up (χ² = 6.35; P < 0.05), whereas the AAT haplotype was higher in the MCNS patients who were completely resolved after 5 years of follow-up (χ² = 5.98; P < 0.015) and in MCNS patients who have completely resolved after 5 years of follow-up (χ² = 6.35; P < 0.012), whereas the AAT haplotype was higher in the MCNS patients who were still relapsing after 5 years of follow-up (χ² = 5.38; P < 0.02). These results suggest that IL-13 genetic polymorphisms, particularly those in the 3′UTR, correlate with the long-term outcome of MCNS in Singapore Chinese children.

As suggested in previous studies, polymorphisms in 3′UTR might be associated with the stability of mRNA. Hence, to study the functional relevance of these IL-13 haplotypes, we randomly recruited five
patients with haplotype GCC and five with haplotype AAT to compare the level of IL-13 mRNA expression in these two groups. In order to exclude the direct effect of steroids as well as abnormalities associated with the relapse state, only those patients in remission and off steroid treatment at the time of blood sampling were included. As shown in Figure 1, the IL-13 mRNA expression index was significantly higher in MCNS patients with haplotype AAT (0.74±0.06) than in those with haplotype GCC (0.38±0.10; P<0.05), suggesting that polymorphisms in the 3'UTR of the IL-13 gene could affect mRNA expression levels.

**Discussion**

In this study, we screened all four exons and flanking introns, the 1729 bp 5' promoter region and 765 bp 3'UTR of the IL-13 gene for SNPs in Singapore Chinese children, and found six previously described SNPs located in the promoter region (704 and 1103), exon 4 (4257) and 3'UTR (4738, 4793 and 4962). IL-13 has been recognized as an important and unique mediator of atopy, and genetic variants of the IL-13 gene have been associated with childhood atopy, asthma or high serum IgE levels.

We and others have provided circumstantial evidence that a predominance of Th2 cytokine responses occur in children with steroid-responsive nephrotic syndrome, involving either IL-13 or IL-4 [4,6] and their respective receptors, IL4R and IL13R [7]. Moreover, MCNS has a well-recognized association with atopy. Various studies have described a higher incidence of allergic disorders, such as asthma, allergic rhinitis and atopic eczema, in children with steroid-responsive nephrotic syndrome. Although immunological studies in these children have been somewhat variable, some studies did report higher mean serum IgE levels in children with steroid-responsive nephrotic syndrome when compared with controls [19]. It is, thus, reasonable to assume that genetic variants of the IL-13 gene may be associated with childhood nephrotic syndrome.

However, studies in different populations have not been supportive of an association between IL-13 genetic polymorphisms and predisposition to MCNS. Although the 1103 (C/T) polymorphism in the promoter region of the IL-13 gene has been shown to be associated with allergic asthma in a Dutch population [13,17], Gillespie et al. [20] did not find any association with MCNS in a British Caucasian population. Similarly, Tenbrock et al. [14] were unable to demonstrate any significant difference in the frequency of the polymorphism R130Q in exon 4 between cases and controls and between the different courses of nephrotic syndrome in 84 German children with MCNS. In the present study, we have shown the presence of six SNPs in the IL-13 gene in Singapore Chinese children. However, there was no difference in both genotype and allele frequencies of any these SNPs between children with MCNS and normal non-atopic controls or children with allergic asthma (unpublished data). Additionally, there was no significant difference in allele frequencies of these SNPs in MCNS children with and without evidence of clinical atopy. Hence, data in both Asian and Caucasian populations suggest that there is no association of IL-13 gene polymorphisms with susceptibility to MCNS.
In view of the heterogeneity in the patients’ clinical course, we further stratified the patients in terms of steroid-responsiveness, steroid-dependency and long-term outcome, and examined their association with IL-13 gene polymorphisms. To date, no clinical predictors of long-term outcome have been described in children with MCNS. Follow-up of our cohort of children with biopsy-proven MCNS showed that none of the clinical features at first presentation, such as age of onset, haematuria, hypertension and serum creatinine levels, as well as histological parameters, such as IgM deposits, were associated with long-term outcome (data not shown).

In this study, there was no significant association between IL-13 gene polymorphisms and steroid-responsiveness or steroid-dependence. However, with regards to long-term outcome, comparing those children who had completely remitted and those who were still relapsing after ≥5 years of follow-up, there was a significant difference in the allele frequencies of the three SNPs in the 3′UTR in tight linkage disequilibrium with each other in the Singapore Chinese children. Moreover, haplotype analysis of the three 3′UTR SNPs showed that both normal controls and MCNS patients who had achieved complete remission in the long-term had a significant excess of the ‘protective’ GCC haplotype, compared with children whose disease was still relapsing. Conversely, children who were still relapsing had an excess of the ‘at risk’ AAT haplotype, compared with normal controls and children in complete remission.

Polymorphisms in the IL-13 coding region have been associated with increased serum IgE levels, whereas IL-13 promoter polymorphisms are associated with altered regulation of IL-13 production and increased binding of nuclear proteins. However, the functional significance of the 4738 (G/A), 4793 (C/A) and 4926 (C/T) polymorphisms in the 3′UTR of the IL-13 gene is unknown. We have demonstrated previously that IL-13 mRNA expression was significantly increased in T cells of nephrotic patients in relapse, compared with remission and normal controls [4]. In this study, we have shown that IL-13 mRNA expression was increased in MCNS patients with the ‘at risk’ AAT haplotype. In some cytokine genes, polymorphisms of the 3′UTR are related to the stability of the mRNA and post-translational efficiency. It is, hence, tempting to postulate that the association of these polymorphisms within the 3′UTR of the IL-13 gene with long-term outcome induces a potential ‘switch’ or age-related decrease in translational efficiency affecting cytokine expression.

In conclusion, we have identified six SNPs in the IL-13 gene in Singapore Chinese children. Although none of these polymorphisms were associated with increased disease susceptibility to MCNS, the SNPs in the 3′UTR were predictive of long-term outcome of the disease.

Acknowledgements. The authors thank Dr Wei-Kin Gong for recruiting patients into this study, and Dr Denise L.M. Goh and Dr Lynette P.C. Shek for sharing the unpublished data on the patients with allergic asthma. This paper was presented in part at the 36th Annual Meeting of the American Society of Nephrology, 2003. This work was supported by grants from the National Medical Research Council, Singapore (NMRC/0562/2001) and Academic Research Fund, National University of Singapore (R-178-000-058-214).

Conflict of interest statement. None declared.

References


Received for publication: 20.5.04
Accepted in revised form: 19.11.04