The CTLA-4 +49GG genotype is associated with susceptibility for nephrotic kidney diseases

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ABSTRACT

Background. The pathogenesis of primary nephrotic kidney diseases is not completely understood. As T-cell involvement is suspected, cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed on activated T cells could play a role in the immune response. Single-nucleotide polymorphisms (SNPs) in the CTLA-4 gene are associated with several autoimmune-related diseases.

Methods. Our goal was to study the occurrence of the SNPs −318C/T, +49A/G and CT60 on the CTLA-4 gene in healthy blood donors (N = 156) compared with nephrotic patients with biopsy-proven minimal-change disease (MCD, N = 160), focal segmental glomerulosclerosis (FSGS, N = 159) and membranous nephropathy (MN, N = 185). Odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated to estimate the strength of the association.

Results. The +49GG genotype was significantly (P < 0.001) associated with the risk for MCD, FSGS and MN (AA versus GG: OR = 3.403, 95% CI = 1.748–6.622, OR = 3.846, 95% CI = 1.945–7.604 and OR = 2.381, 95% CI = 1.257–4.511, respectively). No further significant associations, neither with the heterozygous genotype of +49A/G nor for the −318C/T or CT60 SNP, were detected.

Conclusions. The +49GG genotype of the +49A/G SNP in the CTLA-4 gene is associated with the risk for MCD, FSGS and MN, suggesting a possible role for CTLA-4 in a proposed common final pathway in the pathogenesis of primary nephrotic kidney diseases.
Since CTLA-4 chimeric protein provided effective inhibition in B7-dependent T-cell activation with an improvement in rat autoimmune anti-glomerular basement membrane glomerulonephritis [9] and decreased renal allograft rejection in rats [10], CTLA-4 has become an interesting target molecule for renal diseases.

Many single-nucleotide polymorphisms (SNP), e.g. −318C/T, +49A/G and CT60 (−6230G/A), have been identified for the CTLA-4 gene [11]. For the −318T/T and +49AA genotype, increased expression of cell-surface CTLA-4 and CTLA-4 mRNA was detected leading to the hypothesis of a decreased risk for the susceptibility of autoimmune diseases for these genotypes [12, 13], while the +49GG genotype was associated with decreased expression of CTLA-4 [14]. Furthermore, the +49GG genotype and the G allele were more common in patients with renal involvement in paediatric Henoch-Schönlein purpura [15]. For the CT60 polymorphism, a decreased susceptibility for rheumatoid arthritis has also been described in the Chinese Han population [17].

Since an interaction of podocyte antigens, dendritic cells and T-cells have been shown to contribute to renal injury in two mouse models with profound proteinuria [18, 19], factors of T-cells have been shown to contribute to renal injury in two mouse models with profound proteinuria [18, 19], factors which are involved in T-cell activation seem to be a worthwhile target for further studies in nephrotic renal diseases. Therefore, the goal of this study was to investigate the possible role of the CTLA-4 SNPs −318C/T (5’ regulatory region), +49A/G (exon 1) and CT60 (3’ untranslated region) for the susceptibility of the nephrotic renal diseases MCD, FSGS and MN.

**Isolation of genomic DNA**

Blood samples were collected from buffy coats of 156 healthy Caucasian blood donors serving as a control group. Genomic DNA was extracted as described earlier [20]. Thirty micrometre slices from paraffin-embedded kidney biopsy samples from 160 MCD, 159 FSGS and 185 MN patients of Caucasian origin with nephrotic-range proteinuria were provided as a kind gift of Udo Helmchen, Nierenregister at UKE. and genomic DNA was isolated as described earlier [20]. This study was approved by the ethics committee of the physiicians’ board of the city of Hamburg and has been conducted according to the Declaration of Helsinki.

**SNP genotyping**

The identification of the three CTLA-4 gene polymorphisms −318C/T (rs5742909), +49A/G (rs231775) and CT60 (rs3087243) was carried out using PCR, followed by a specific restriction enzyme digest for each SNP. The resulting products were separated by electrophoresis in 2% agarose gels, containing ethidium bromide in a final concentration of 0.5 µg/mL. For the −318C/T SNP, the flanking primer pair 5’-GGGATTCTTAGGGACCCCTTG-3’ (forward) and 5’-GTGCACACACACGAGGACCT-3’ (reverse) was used resulting in a 244-bp fragment. For the +49A/G SNP, primers were 5’-GGCTTGCCCTGTCC-3’ (forward) and 5’-CCCAGTGAGGGAACACCTC-3’ (reverse) resulting in a 160-bp fragment; and for the CT60 SNP, the respective primers were 5’-TTGGATTCTAGGGAAAACCTC-3’ (forward) and 5’-AGGGAGGATGGGAACACCTC-3’ (reverse) resulting in a 163-bp fragment.

One to five microlitres of genomic DNA were added to 2 µL of Dream-Taq buffer (Fermentas, St. Leon-Rot, Germany), 0.4 µL of dNTPs (10 mM each), 1.0 µL of the respective forward and of the respective reverse primer (10 pmol/µL each), 0.2 µL of Dream-Taq polymerase (5 U/µL) and 11.4-7.4 µL of H2O, respectively, resulting in a total volume of 20 µL. PCRs were run for 36 cycles using the following temperature profiles: initialization at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 58°C (for C-318 T and CT60) or 56°C (for A49G), extension at 72°C for 60 s, followed by a single final extension step at 72°C for 5 min.

For restriction digests, Msel (10 U/µL) was used for −318C/T, ApekI (4 U/µL) for +49A/G and HpyCH4IV (10 U/µL) for CT60. All restriction enzymes were purchased from New England Biolabs, Frankfurt/Main, including their specific buffers. Total reaction volume for all digests was 25 µL. Digest protocol for −318CT: 10.0 µL PCR product, 0.5 µL Msel, 1.25 µL NEBuffer 2 (10×), 0.5 µL bovine serum albumin (100×), 12.75 µL H2O, incubation over night at 37°C. Digest protocol for +49A/G: 10.0 µL PCR product, 0.2 µL ApekI, 0.5 µL NEBuffer 3 (10×), 14.3 µL H2O, incubation over night at 75°C. Digest protocol for CT60: 10.0 µL PCR product, 0.2 µL HpyCH4IV, 0.5 µL NEBuffer 1 (10×), 14.3 µL H2O, incubation over night at 37°C. Msel cuts the 244-bp PCR product of −318C/T when the TT genotype is present, resulting in a 146- and 98-bp band. Heterozygous samples show three bands (244, 146 and 98 bp), whereas the homozygous CC genotype only reveals an undigested 244-bp band. The 160-bp PCR fragment of +49A/G is cut by ApekI when the GG genotype is present, resulting in a 117- and 43-bp fragment. Three bands (160, 117 and 43 bp) are present in the heterozygous state, and a single, undigested band of 160 bp can be found for the homozygous AA genotype. When the homozygous GG genotype of CT60 is present, the 163-bp PCR product of CT60 is digested by HpyCH4IV, resulting in a 87- and 76-bp band. Three bands (163, 87 and 76 bp) are found in heterozygous samples, and a single, undigested 163-bp band for the homozygous AA genotype.

**Statistical analysis**

Allelic and genotypic frequencies were obtained by direct counting. Statistical comparison of genotypic frequencies between MCD, FSGS and MN patients and healthy controls was carried out by the χ2 test. The study was designed to have a power of 0.80 to detect a difference between two groups of P ≤ 0.05 based on allele frequencies derived from a pre-test with 25 controls and 10 samples from each disease. Odds ratios (ORs) and 95% confidence intervals (CIs) are provided for the distribution of allele and genotype frequencies of the three SNPs, respectively. As in other genetic studies, only statistical differences with P < 0.01 were considered to be relevant.
RESULTS

Age and gender distribution of controls and patients with MCD, FSGS and MN are provided in Table 1. A significant age difference was only observed between healthy controls and patients with MN (P < 0.001). The distribution of men and women showed no significant differences between the four groups.

A sample of the +49A/G analysis is shown in Figure 1A. For the +49A/G SNP, significant differences were found for the occurrence of the G-allele and the +49GG genotype for all three nephrotic diseases compared with controls (Table 2). The +49GG genotype was significantly associated with the risk for MCD (AA versus GG: OR = 3.403, 95% CI = 1.748–6.622, \( \chi^2 = 13.41, P < 0.001 \)), FSGS (AA versus GG: OR = 3.846, 95% CI = 1.945–7.604, \( \chi^2 = 15.58, P < 0.001 \)) and MN (AA versus GG: OR = 2.381, 95% CI = 1.257–4.511, \( \chi^2 = 7.20, P = 0.007 \)). G-allele frequencies were also significantly higher for the three nephrotic diseases (MCD: OR = 1.833, 95% CI = 1.324–2.537, \( \chi^2 = 13.43, P < 0.001 \); FSGS: OR = 1.874, 95% CI = 1.361–2.580, \( \chi^2 = 14.93, P < 0.001 \); MN: OR = 1.494, 95% CI = 1.103–2.024, \( \chi^2 = 6.73, P = 0.009 \)).

A sample of the −318C/T digest is provided in Figure 1B, and the results for the −318C/T analysis are summarized in Table 3. No other significant differences were found for MN or MCD and FSGS. For the CT60 SNP, a sample analysis is shown in Figure 1C, and no significant association was found for any of the nephrotic kidney diseases compared with healthy controls (Table 4).

DISCUSSION

The occurrence of the +49GG genotype in the CTLA-4 gene was associated with an increased susceptibility for all three nephrotic diseases.
nephrotic diseases (MCD, FSGS and MN). No significant differences between the three diseases were found despite patients in the MN group being significantly older due to a higher incidence of this disease in older individuals. The G-allele in position +49 of exon 1 has been shown to affect the CTLA-4-driven down-regulation of T-cell activation negatively [14], leading to the hypothesis that its presence might play an important role in the pathogenesis of autoimmune diseases. As a matter of fact, the presence of the +49 G allele is associated with autoimmune diseases like rheumatoid arthritis [17], Graves' disease [21], primary biliary cirrhosis [22] and an increased risk for renal involvement in Henoch-Schönlein purpura [15]. Also, the interaction of podocyte antigens, dendritic cells and T cells contributes to the large proteinuria in two mouse models of renal injury [18, 19], supporting the hypothesis of a ‘two-hit’ podocyte immune disorder for nephrotic renal diseases as it has been suggested for MCD patients with defective CD80 regulation by podocyte-derived CTLA-4 [7].

The +49 G allele leads to the replacement of threonine by alanine, which might induce a conformational change of the signal peptide of the CTLA-4 protein leading to an insufficient

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<th>Table 2. Allele and genotype frequencies of SNP +49A/G</th>
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<tr>
<td>Controls (N = 156) MCD (N = 142) FSGS (N = 152) MN (N = 185)</td>
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<tr>
<td>+49A/G</td>
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<tr>
<td>AA, n (%)</td>
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<td>AG, n (%)</td>
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<td>GG, n (%)</td>
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Allele frequency

| A | 0.583 | 0.433 | 0.428 | 0.484 |
| G | 0.417 | 0.567* | 0.572* | 0.516* |

Significant differences are marked in bold numbers, *P < 0.001, †P < 0.01

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<th>Table 3. Allele and genotype frequencies of SNP -318C/T</th>
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<tr>
<td>Controls (N = 154) MCD (N = 152) FSGS (N = 150) MN (N = 165)</td>
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<tr>
<td>-318C/T</td>
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<tr>
<td>CG, n (%)</td>
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<tr>
<td>CT, n (%)</td>
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<td>TT, n (%)</td>
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Allele frequency

| C | 0.938 | 0.905 | 0.943 | 0.897 |
| T | 0.062 | 0.095 | 0.057 | 0.103 |

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<th>Table 4. Allele and genotype frequencies of SNP CT60</th>
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<td>Controls (N = 153) MCD (N = 160) FSGS (N = 159) MN (N = 164)</td>
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<td>CT60</td>
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<tr>
<td>AA, n (%)</td>
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<td>AG, n (%)</td>
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<td>GG, n (%)</td>
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Allele frequency

| A | 0.422 | 0.412 | 0.453 | 0.415 |
| G | 0.578 | 0.588 | 0.547 | 0.585 |
transport of CTLA-4 to the cell surface that is important for the down-regulation of the T-cell response [23]. A higher ratio of cell surface/total CTLAThr(17) versus CTLAAla(17) has been observed by confocal microscopy, which suggests an inefficient processing of the CLTA-4 protein when the G allele is present [24]. Consequently, this results in persistence of CD80 expression in T cells and/or podocytes, which could be a ‘second hit’ in nephrotic kidney diseases in addition to other primary pathogenic mechanisms, e.g. the occurrence of phospholipase A2 receptor antibodies in MN that has recently been described [25].

For the −318C/T SNP, no significant differences in genotypes were found for any of the nephrotic diseases. The homozygous −318T/T genotype, which has been shown to be associated with a stimulated expression of CTLA-4 [12], occurs only very occasionally in our nephrotic patients or healthy controls. Even though the heterozygous −318C/T genotype has been shown to be associated with susceptibility for chronic obstructive pulmonary disease [26], heterozygosity for a CTLA-4 mutation had no significant pathological consequences in mice [27]. We did not find any significant differences for the CT60 SNP for any of the three nephrotic renal diseases compared with controls.

Our study has several limitations. It only included Caucasian patients and patient numbers in the different groups are slightly variable due to changes in DNA quality for the PCRs. Due to the age of the patients and the lack of parental DNA, we were not able to perform linkage analyses and haplotype studies for the three SNPs, which might have lead to further insights. Further investigations on a possible linkage of SNP genotypes −318C/T and +49AG should be performed as different levels of CTLA-4 expression and intracellular transport are associated with other disease activities [12].

To our knowledge, this is the first study that compares the occurrence of SNPs in the CTLA-4 gene in different nephrotic renal diseases. In conclusion, the +49GG genotype of the CTLA-4 gene was associated with a significant susceptibility for MCD, FSGS and MN. We thank Melanie Scharper for her excellent technical assistance.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

Screening for albuminuria with subsequent screening for hypertension and hypercholesterolaemia identifies subjects in whom treatment is warranted to prevent cardiovascular events

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\textbf{ABSTRACT}

\textbf{Background.} In the general population, many subjects have yet unrecognized hypertension and hypercholesterolaemia, and are thus not treated. We investigated whether population screening for elevated albuminuria can identify subjects with previously unrecognized hypertension and/or hypercholesterolaemia at high risk for cardiovascular (CV) disease.

\textbf{Methods.} Included were 8143 subjects (28–75 years) that participate in the PREVEND study, a general population-based, observational cohort study. Elevated albuminuria was defined as an albumin concentration \( \geq 20 \text{mg/L} \) in a first morning urine sample confirmed by an albumin excretion \( \geq 30 \text{mg/day} \) in two 24-h urine samples. Hypertension was defined as SBP \( \geq 140 \text{mmHg} \) or DBP \( \geq 90 \text{mmHg} \), and hypercholesterolaemia as serum total cholesterol \( \geq 6.2 \text{mmol/L} \), or as HDL cholesterol \( < 0.9 \text{mmol/L} \) and a total/HDL cholesterol ratio of

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