Kidney & Blood Pressure Research

Kidney Blood Press Res 2018;43:360-366

DOI: 10.1159/000488069
Published online: March 13, 2018

Accepted: March 2, 2018

Original Paper

CTLA-4 Polymorphisms in Patients with IgA Nephropathy Correlate with Proteinuria

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Key Words
CTLA-4 • IgA nephropathy • IgAN • STOP-IgAN • Single nucleotide polymorphism

Abstract
Background/Aims: IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis and still constitutes one of the most important causes of end-stage renal disease. Abnormal T cell responses may play a role in IgAN pathogenesis. Co-stimulatory molecules such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) are important for naive T cells to initiate and terminate immune responses. Single nucleotide polymorphisms (SNPs) in the CTLA4 gene locus are associated with several autoimmune diseases. ***Methods:*** We aimed to investigate the occurrence of the SNPs -318C/T, +49A/G and CT60 G/A within the CTLA4 locus in healthy blood donors (n=455) and IgAN patients (n=252) recruited from the recently published STOP-IgAN trial. The presence of these SNPs was then associated with baseline proteinuria in IgAN patients. ***Results:*** We observed a significantly increased frequency of the CTLA4 -318C/T genotype in IgAN patients as compared to controls (CC vs. CT+TT: OR 1.65, 95%-CI 1.03-2.65, p=0.035). No significant associations, neither with the +49A/G nor for the CT60 G/A SNP, were detected. However, when we stratified for proteinuria at time of inclusion into the STOP-IgAN trial (<1 g/day vs. >1 g/day), we observed significant differences in the frequencies of the CT60 G/A genotype, i.e. a significantly increased risk for higher proteinuria in patients carrying the G allele (OR 2.81, 95%-CI 1.03-7.64, p=0.042). ***Conclusion:*** The CTLA4 -318/C/T SNP was associated with an increased risk to develop IgAN, while the CT60 G/A genotype significantly associated with the risk for higher proteinuria suggesting a possible role for CTLA-4 in IgAN.

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Introduction

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis and still constitutes one of the most important causes of end-stage renal disease [1, 2]. The immunopathogenesis of IgAN involves an aberrant O-galactosylation of IgA1 molecules, production of O-glycan-specific autoantibodies and formation of nephritogenic immune complexes that deposit in the glomerular mesangium and evoke glomerular injury [3]. In addition, an abnormal T cell response has also been suggested in pathogenesis of IgAN and co-stimulatory molecules such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) have been identified to be important for naive T cells to initiate and terminate immune responses [4, 5]. In particular, CTLA4 polymorphisms have been described in Schoenlein-Hennoch Nephritis, which is an IgA-related nephropathy that mainly occurs in children [6].

Recent genome-wide association studies (GWAS) unravelled a number of IgAN susceptibility genes that relate to components of the intestinal mucosa barrier, the O-glycosylation pathway and the complement system [7-11]. Against the background of these findings, it is astonishing that most IgAN patients do not need or benefit from immunosuppressive treatment, whereas the value of comprehensive supportive measures is undisputed in IgAN patients. Findings from our Supportive versus Immunosuppressive Therapy for the Treatment of Progressive IgAN (STOP-IgAN) trial did not support the use of immunosuppression on top of optimized supportive care since added immunosuppression did not stabilize renal function better than supportive care alone [12].

In the present study, we investigated three single nucleotide polymorphisms (SNPs) at the cytotoxic T lymphocyte-associated *antigen* 4 (CTLA4) gene locus. CTLA-4 is mainly expressed on T lymphocytes and represents an essential negative regulator of the immune system. CTLA-4 has been implicated to play a role in a number of autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, Grave's disease and multiple sclerosis [13, 14].

We recently demonstrated that one of these SNPs (+49A/G) significantly correlates with nephrotic diseases in adults and children including minimal change disease (MCD), focal segmental glomerulosclerosis and membranous glomerulonephritis [15, 16]. Additionally, these SNPs have been shown to affect the expression of either membrane bound or soluble CTLA-4.

CTLA-4 belongs to the immunoglobin superfamily and is expressed as a cell surface co-receptor on T cells. Both, CD80 and CD86, that are present on antigen-presenting cells, act as ligands for CTLA4. In contrast to CD28, CTLA4 acts in antagonistic manner and inhibits T cell activation [17]. Markedly enhanced urinary CD80 excretion has been described in children and adults with nephrotic syndrome during relapse [18]. CD80 expression on podocytes, which can be induced by TLR4 activation, is associated with enhanced proteinuria and podocyte foot process effacement [19]. In IgAN, CD86 is predominantly expressed in the glomeruli and in the peritubular interstitium and CD80/CD86 expression levels correlate with renal function at the time of renal biopsy. Most $CD86^+$ cells are monocytes and macrophages [20].

Materials and Methods

Isolation of genomic DNA

DNA was isolated from EDTA blood drawn from 252 patients with biopsy-proven IgA nephropathy recruited from the STOP-IgAN trial [12]. Additionally, DNA was isolated from buffy coats of 455 healthy Caucasian blood donors serving as control group. The control group consisted of 299 blood donors from the blood bank of the RWTH Aachen and of 156 healthy blood donors from the UKE Hamburg. This study was approved by the Ethics Committees of the Physicians' Board of Hamburg and Aachen and has been conducted according to the Declaration of Helsinki.

SNP genotyping

The identification of the three CTLA4 gene polymorphisms -318/C/T (rs5742909; NM_001037631.2:c.–318C>T), +49A/G (rs231775; NM_001037631.2:c.49A>G) and CT60 G/A (rs3087243; NM_001037631.2:c.*1421G>A; also referred to as CT60 G/A) was carried out using PCR, followed by 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 -318/C/T SNP the flanking primer pair 5'-GGGATTTAGGAGGACCCTTG-3' (forward) and 5'-GTGCACACACAGAAGGCACT-3' (reverse) was used resulting in a 244 bp fragment. For the +49A/G SNP primers were 5'-GGCTTGCCTTGGATTTCA G-3' (forward) and 5'-CCCAGGTAGGAGAAACACCTC-3' (reverse) resulting in a 160 bp fragment, and for the CT60 G/A SNP the respective primers were 5'-CTTTGCACCAGCCATTACCT-3' (forward) and 5'-AGGGGAGGTGA GAACCTGT-3' (reverse) resulting in a 163 bp fragment.

One to five microliters of genomic DNA were added to 2 µl of Dream-Taq buffer (Fermentas, St. Leon-Rot), 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 Dream-Taq polymerase (5 U/µl), and 11.4 to 7.4 µl of H_2O, 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 -318/C/T and CT60 G/A) or 56°C (for +49A/G), extension at 72°C for 60 s, followed by a single final extension step at 72°C for 5 min.

For restriction digests MseI was used for -318C/T, ApeKI for +49A/G, and HpyCH4IV for CT60 G/A. MseI cuts the 244 pb PCR product of -318/C/T when the TT genotype is present, resulting in a 146 bp and a 98 bp band. Heterozygous samples show three bands (244 bp, 146 bp 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 bp and a 43 bp fragment. Three bands (160 bp, 117 bp 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 G/A is present the 163 pb PCR-product of CT60 G/A is digested by HpyCH4IV, resulting in a 87 bp and a 76 bp band. Three bands (163 bp, 87 bp and 76 bp) are found in heterozygous samples and a single, undigested 163 bp band for the homozygous AA genotype.

Statistical analysis

Genotypic frequencies were obtained by direct counting. Statistical comparison of genotypic frequencies between IgAN patients and healthy controls was carried out by $\chi 2$ test. Odds ratios (ORs) and 95% confidence intervals (CIs) are provided for the distribution of allele and genotype frequencies of the three SNPs, respectively.

Results

To further explore a pathogenic role for CTLA-4 in IgAN, we analyzed the presence of three single nucleotide polymorphisms (SNPs) at the CTLA4 gene locus (denoted -318/C/T, +49A/G and CT60 G/A, schematically depicted in Fig. 1) by PCR-RFLP in 252 patients with biopsy-proven IgAN recruited from the STOP-IgAN cohort (patient characteristics of the present subcohort at the time of enrollment into the trial: 75% men, mean age: 43.2 ± 12.6 years, mean GFR 75 ± 35.4 ml/min/1.73 m^2, mean proteinuria: 2.2 ± 1.7 g/day, blood pressure around 131/81 mmHg) and 455 healthy controls. We observed a significantly increased frequency of the CTLA4 -318/C/T SNP in IgAN patients as compared to controls

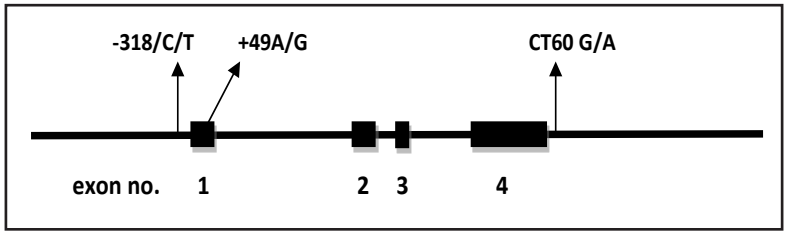

Fig. 1. Scheme with localization of the -318/C/T, +49A/G and CT60 G/A polymorphisms within the human CTLA4 gene.

Table 1. Absolute and relative genotype frequencies of investigated polymorphisms

CTLA4 SNP	Patients (%)	Controls (%)	OR (95%-CI)	p-value
-318/C/T	(n=249)	(n=315)		
CC	204 (81.9)	278 (88.2)		
CT	42 (16.8)	34 (10.8)		
TT	3 (1.2)	3 (0.9)		
CT/TT	45 (18)	37 (11.7)	1.65 (1.03-2.65)	0.035
+49A/G	(n=245)	(n=455)		
AA	105 (42.8)	177 (38.9)		
AG	113 (46)	215 (47.2)		
GG	27 (11)	63 (13.8)	1.17 (0.85-1.61)	0.33
CT60 G/A	(n=244)	(n=436)		
AA	53 (23.7)	99 (22.7)		
AG	133 (54%)	207 (47.5)		
GG	58 (21.7)	130 (29.8)	0.94 (0.64-1.37)	0.77

Table 2. Absolute and relative genotype frequencies of +49 AG and CT60 G/A polymorphisms with regard to proteinuria (at inclusion into the STOP-IgAN trial)

CTLA4 SNP	Proteinuria >1 g/d (%)	Proteinuria <1 g/day (%)	OR (95%-CI)	p-value
+49A/G	(n=173)	(n=53)		
AA	72 (41.6)	27 (50.9)		
AG	77 (44.5)	24 (45.2)		
GG	24 (13.8)	2 (3.7)	GG vs. AA 4.5 (0.99-20.06)	0.0507
	(n=173)	(n=58)		
#	35 (26.5)	15 (25.8)		
	92 (53.2)	36 (62)		
	46 (20)	7 (12)	GG vs. AA 2.81 (1.03-7.64)	0.042

(CT+TT vs. CC: OR 1.65, 95%-CI 1.03-2.65, p=0.035; Table 1). Also, in the allele frequency model (T vs. C) there was a significant difference between patients and controls (OR 1.57, 95%-CI 1.01-2.43, p=0.04).

In contrast to the data in patients with MCD and Schoenlein Henoch nephritis [6, 16], we did not observe statistically significant differences in the frequencies of the +49A/G and CT60 G/A G/A SNPs compared to controls. However, when we stratified for proteinuria at time of inclusion into the STOP-IgAN trial (<1 g/day vs. >1 g/day), we observed relevant differences in the frequencies of these two polymorphisms (Table 2). The +49A/G genotype associated with the risk for higher proteinuria (defined as >1 g/day) (GG vs. AA: OR 4.5, 95%-CI 0.99-20.6, p=0.0507) and tends to confirm our data from adults with pediatric patients and adults with MCD [15, 16]. In IgAN patients, the CT60 G/A genotype even significantly associated with the risk for higher proteinuria in the homozygous model by comparing GG vs. AA. Patients carrying the G allele had a 2.81-fold increase risk to develop higher proteinuria (95%-CI 1.03-7.64, p=0.042).

All analyzed SNPs did not associate with GFR at the time of inclusion into the study or any of the two co-primary endpoints of the main STOP-IgAN trial, i.e. achievement of full clinical remission and eGFR-loss ≥15 ml/min/1.73 m^2, and ESRD occurrence over the 3-year trial phase (data not shown).

Discussion

In this study, we analyzed the presence of the CTLA4 SNPs -318/C/T (within the 5' regulatory region), +49A/G (exon 1) and CT60 G/A (3' untranslated region) in IgAN patients. At the -318/C/T site, we link both, the presence of the CT/TT genotypes and the T allele, within the CTLA4 locus with a significantly increased susceptibility to develop IgAN. The frequency of this polymorphism was not significantly altered in both, our adult and pediatric group with MCD compared to controls and the C allele is known to associate with lower CTLA-4 expression. Thus, we find a higher abundance of the T allele in IgAN patients which

is associated with enhanced CTLA-4 expression and would suggest an immunosuppressive phenotype [21]. However, the overall abundance of the T allele is rather low in the general and in the IgAN population (3% homozygous, 16% heterozygous).

Second, we find that the frequencies of the other two polymorphisms within the CTLA4 gene (+49A/G and CT60 G/A), which result in suppressed CTLA-4 expression and thereby exert inhibitory function, are not altered between healthy controls and IgAN patients. However, when we stratified for proteinuria at inclusion into the STOP-IgAN trial, the CT60 G/A and +49A/G genotypes that result in down-regulated CTLA-4 expression associate with higher baseline proteinuria and corroborate findings from adults and children with MCD and other glomerulopathies [15, 16]. The +49 G allele results in replacement of threonine by alanine which possibly induces a conformational change of CTLA-4 leading to an inefficient processing and reduced CTLA-4 surface expression. Eventually, this might result in impaired T-cell responses [22]. Enhanced frequency of this polymorphism has also been identified as a risk factor to develop Schoenlein Henoch nephritis [6].

The CT60 G/A SNP confers an aberrant splicing of the CTLA4 gene. The G allele was found to be increased in IgAN patients with higher proteinuria and results in down-regulation of the soluble form of CTLA-4 as compared to its membrane-bound full-length counterpart. This polymorphism has been associated with a number of autoimmune diseases including Grave's disease, diabetes and arthritis [13].

Thus, with respect to the CT60 G/A and +49A/G polymorphisms our data suggest that SNPs at these sites that mediate a decreased CTLA-4 expression might constitute an independent risk factor to aggravate proteinuria in IgAN patients.

Of note, we did not detect any association between the SNP frequencies in the included IgAN cohort, that was recruited from the recently published STOP-IgAN trial [12], and major trial endpoints such as full clinical remission, eGFR-loss of at least 15 ml/min/1.73 m^2 and ESRD occurrence. This might be explained by the relatively short follow-up time of 3 years only. Given the slow disease progression of IgAN in most cases, differences might only become apparent during longer observation periods. Another limitation of the present analysis relates to the inclusion of solely Caucasian IgAN patients. Since IgAN presents in a highly variable disease pattern with significant differences in the genetic background, disease progression and treatment responses, CTLA4 SNPs should be analyzed in larger cohorts of multiethnic origins.

Our data suggest that CTLA-4 may be involved in IgAN development and disease progression since frequencies of the -318/C/T SNP within the CTLA4 gene locus significantly differ between IgAN patients and healthy controls. Furthermore, the presence of the CTLA4 SNPs +49A/G and CT60 G/A were associated with higher proteinuria (> 1 g/day) in IgAN patients. Future studies evaluating expression levels of CTLA-4 on mononuclear cells and in the affected kidneys will help to elucidate the role of CTLA-4 in IgAN and whether it might serve as a potential therapeutic target.

Disclosure Statement

There is no conflict of interest regarding this manuscript.

Acknowledgements

Authors' Contributions: Marius Jacob, performed experiments and wrote the paper; Kim Ohl, performed experiments and wrote the paper; Tannaz Goodarzi, performed experiments; Sigrid Harendza, contributed Hamburg control cohort; Thomas Eggermann, contributed Aachen control cohort; Christina Fitzner, performed statistics; Ralf-Dieter Hilgers, performed statistics; Anna Bolte, performed analysis of patient data; Jürgen Floege, designed the main

STOP-IgAN trial and contributed cohort; Thomas Rauen, designed the project and wrote the paper; Klaus Tenbrock, designed the project and wrote the paper.

Analyses performed in the present study were funded by Bristol-Myers-Sqibb (BMS).

References

1 Wyatt RJ, Julian BA: IgA nephropathy. N Engl J Med 2013;368:2402-2414.
2 Rodrigues JC, Haas M, Reich HN: IgA Nephropathy. Clin J Am Soc Nephrol 2017;12:677-686.
3 Rauen T, Floege J: Inflammation in IgA nephropathy. Pediatr Nephrol 2017;32:2215-2224.
4 Carreno BM, Carter LL, Collins M: Therapeutic opportunities in the B7/CD28 family of ligands and receptors. Curr Opin Pharmacol 2005;5:424-430.
5 Carreno BM, Collins M: The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol 2002;20:29-53.
6 Wang JJ, Shi YP, Yue H, Chun W, Zou LP: CTLA-4 exon 1 +49A/G polymorphism is associated with renal involvement in pediatric Henoch-Schonlein purpura. Pediatr Nephrol 2012;27:2059-2064.
7 Feehally J, Farrall M, Boland A, Gale DP, Gut I, Heath S, Kumar A, Peden JF, Maxwell PH, Morris DL, Padmanabhan S, Vyse TJ, Zawadzka A, Rees AJ, Lathrop M, Ratcliffe PJ: HLA has strongest association with IgA nephropathy in genome-wide analysis. J Am Soc Nephrol 2010;21:1791-1797.
8 Gharavi AG, Kiryluk K, Choi M, Li Y, Hou P, Xie J, Sanna-Cherchi S, Men CJ, Julian BA, Wyatt RJ, Novak J, He JC, Wang H, Lv J, Zhu L, Wang W, Wang Z, Yasuno K, Gunel M, Mane S, et al.: Genome-wide association study identifies susceptibility loci for IgA nephropathy. Nat Genet 2011;43:321-327.
9 Yu XQ, Li M, Zhang H, Low HQ, Wei X, Wang JQ, Sun LD, Sim KS, Li Y, Foo JN, Wang W, Li ZJ, Yin XY, Tang XQ, Fan L, Chen J, Li RS, Wan JX, Liu ZS, Lou TQ, et al.: A genome-wide association study in Han Chinese identifies multiple susceptibility loci for IgA nephropathy. Nat Genet 2011;44:178-182.
10 Kiryluk K, Li Y, Moldoveanu Z, Suzuki H, Reily C, Hou P, Xie J, Mladkova N, Prakash S, Fischman C, Shapiro S, LeDesma RA, Bradbury D, Ionita-Laza I, Eitner F, Rauen T, Maillard N, Berthoux F, Floege J, Chen N, et al.: GWAS for serum galactose-deficient IgA1 implicates critical genes of the O-glycosylation pathway. PLoS Genet 2017;13:e1006609.
11 Kiryluk K, Li Y, Scolari F, Sanna-Cherchi S, Choi M, Verbitsky M, Fasel D, Lata S, Prakash S, Shapiro S, Fischman C, Snyder HJ, Appel G, Izzi C, Viola BF, Dallera N, Del Vecchio L, Barlassina C, Salvi E, Bertinetto FE, et al.: Discovery of new risk loci for IgA nephropathy implicates genes involved in immunity against intestinal pathogens. Nat Genet 2014;46:1187-1196.
12 Rauen T, Eitner F, Fitzner C, Sommerer C, Zeier M, Otte B, Panzer U, Peters H, Benck U, Mertens PR, Kuhlmann U, Witzke O, Gross O, Vielhauer V, Mann JF, Hilgers RD, Floege J: Intensive Supportive Care plus Immunosuppression in IgA Nephropathy. N Engl J Med 2015;373:2225-2236.
13 Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G, Herr MH, Dahlman I, Payne F, Smyth D, Lowe C, Twells RC, Howlett S, Healy B, Nutland S, Rance HE, et al.: Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature 2003;423:506-511.
14 Scalapino KJ, Daikh DI: CTLA-4: a key regulatory point in the control of autoimmune disease. Immunol Rev 2008;223:143-155.
15 Spink C, Stege G, Tenbrock K, Harendza S: The CTLA-4 +49GG genotype is associated with susceptibility for nephrotic kidney diseases. Nephrol Dial Transplant 2013;28:2800-2805.
16 Ohl K, Eberhardt C, Spink C, Zahn K, Wagner N, Eggermann T, Kemper MJ, Querfeld U, Hoppe B, Harendza S, Tenbrock K: CTLA4 polymorphisms in minimal change nephrotic syndrome in children: a case-control study. Am J Kidney Dis 2014;63:1074-1075.
17 Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH: Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995;3:541-547.
18 Cara-Fuentes G, Wasserfall CH, Wang H, Johnson RJ, Garin EH: Minimal change disease: a dysregulation of the podocyte CD80-CTLA-4 axis? Pediatr Nephrol 2014;29:2333-2340.

19 Reiser J, von Gersdorff G, Loos M, Oh J, Asanuma K, Giardino L, Rastaldi MP, Calvaresi N, Watanabe H, Schwarz K, Faul C, Kretzler M, Davidson A, Sugimoto H, Kalluri R, Sharpe AH, Kreidberg JA, Mundel P: Induction of B7-1 in podocytes is associated with nephrotic syndrome. J Clin Invest 2004;113:1390-1397.

20 Wu Q, Jinde K, Endoh M, Sakai H: Clinical significance of costimulatory molecules CD80/CD86 expression in IgA nephropathy. Kidney Int 2004;65:888-896.

21 Ligers A, Teleshova N, Masterman T, Huang WX, Hillert J: CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. Genes Immun 2001;2:145-152.

22 Valk E, Rudd CE, Schneider H: CTLA-4 trafficking and surface expression. Trends Immunol 2008;29:272-279.