

World Journal of *Gastroenterology*

World J Gastroenterol 2020 May 14; 26(18): 2126-2275



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The WJG is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Scopus. The 2019 edition of Journal Citation Report® cites the 2018 impact factor for WJG as 3.411 (5-year impact factor: 3.579), ranking WJG as 35th among 84 journals in gastroenterology and hepatology (quartile in category Q2). CiteScore (2018): 3.43.

RESPONSIBLE EDITORS FOR THIS ISSUE

Responsible Electronic Editor: Yan-Liang Zhang

Proofing Production Department Director: Yun-Xiaojuan Wu

Responsible Editorial Office Director: Ze-Mao Gong

NAME OF JOURNAL

World Journal of Gastroenterology

ISSN

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE

October 1, 1995

FREQUENCY

Weekly

EDITORS-IN-CHIEF

Subrata Ghosh, Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS

<http://www.wjgnet.com/1007-9327/editorialboard.htm>

PUBLICATION DATE

May 14, 2020

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INSTRUCTIONS TO AUTHORS

<https://www.wjgnet.com/bpg/gerinfo/204>

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<https://www.wjgnet.com/bpg/GerInfo/287>

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH

<https://www.wjgnet.com/bpg/gerinfo/240>

PUBLICATION ETHICS

<https://www.wjgnet.com/bpg/GerInfo/288>

PUBLICATION MISCONDUCT

<https://www.wjgnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE

<https://www.wjgnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS

<https://www.wjgnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION

<https://www.f6publishing.com>



Basic Study

Genetic association analysis of *CLEC5A* and *CLEC7A* gene single-nucleotide polymorphisms and Crohn's disease

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Author contributions: Schäffler H, Rohde S and Jaster R designed the study; Huth A, Schäffler H and Lamprecht G took responsibility for patient care and follow-up; Elleisy N, Rohde S and Jaster R performed the experiments; Gittel N, Huth A and Schäffler H collected the samples and performed the clinical characterisation of the patients; Elleisy N, Glass Ä, Möller S and Jaster R performed the biostatistics; all authors analyzed the data; Schäffler H and Jaster R wrote the manuscript and contributed equally and share senior authorship.

Supported by the Damp-Foundation, No. 2016-04.

Institutional review board statement: The study was approved by the ethic board of the Medical Faculty of the University of Rostock (A 2017-0137). Written informed consent was obtained from each participant prior to enrollment.

Conflict-of-interest statement: The authors declare that there is no conflict of interest.

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Abstract

BACKGROUND

Crohn's disease (CD) is characterized by a multifactorial etiology and a significant impact of genetic traits. While *NOD2* mutations represent well established risk factors of CD, the role of other genes is incompletely understood.

AIM

To challenge the hypothesis that single nucleotide polymorphisms (SNPs) in the genes *CLEC5A* and *CLEC7A*, two members of the C-type lectin domain family of pattern recognition receptors, may be associated with CD.

METHODS

SNPs in *CLEC5A*, *CLEC7A* and the known CD risk gene *NOD2* were studied using real time PCR-based SNP assays. Therefore, DNA samples from 175 patients and 157 healthy donors were employed. Genotyping data were correlated with clinical characteristics of the patients and the results of gene expression data analyses.

RESULTS

In accordance with previous studies, rs2066844 and rs2066847 in *NOD2* were found to be significantly associated with CD (allelic *P* values = 0.0368 and 0.0474, respectively). Intriguingly, for genotype AA of rs1285933 in *CLEC5A*, a potential association with CD (recessive *P* = 0.0523; odds ratio = 1.90) was observed. There were no associations between CD and SNPs rs2078178 and rs16910631 in *CLEC7A*. Variants of rs1285933 had no impact on *CLEC5A* gene expression. In contrast, genotype-dependent differences of *CXCL5* expression in peripheral blood mononuclear cells were observed. There is no statistical interaction

Data sharing statement: No additional data are available.

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Manuscript source: Unsolicited manuscript

Received: February 4, 2020

Peer-review started: February 4, 2020

First decision: March 21, 2020

Revised: April 13, 2020

Accepted: April 29, 2020

Article in press: April 29, 2020

Published online: May 14, 2020

P-Reviewer: Mach TH

S-Editor: Tang JZ

L-Editor: A

E-Editor: Zhang YL



between the tested SNPs of *NOD2* and *CLEC5A*, suggesting of a novel pathway contributing to the disease.

CONCLUSION

Our data encourage enlarged follow-up studies to further address an association of SNP rs1285933 in *CLEC5A* with CD. The C-type lectin domain family member also deserves attention regarding a potential role in the pathophysiology of CD.

Key words: Crohn's disease; Single nucleotide polymorphisms; *NOD2*; *CLEC5A*; Gene expression; *CXCL5*

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Core tip: The genetic traits of Crohn's disease (CD) are incompletely understood. Here, we report a potential association of single nucleotide polymorphism (SNP) rs1285933 in *CLEC5A*, a member of the C-type lectin domain family of pattern recognition receptors, with CD. Variants of SNP rs1285933 had no impact on *CLEC5A* gene expression in peripheral blood mononuclear cells but correlated with the expression of *CXCL5*. The SNPs rs2078178 and rs16910631 in *CLEC7A* were not associated with the disease. The role of *CLEC5A* in the pathophysiology of CD deserves further attention.

Citation: Elleisy N, Rohde S, Huth A, Gittel N, Glass A, Möller S, Lamprecht G, Schäffler H, Jaster R. Genetic association analysis of *CLEC5A* and *CLEC7A* gene single-nucleotide polymorphisms and Crohn's disease. *World J Gastroenterol* 2020; 26(18): 2194-2202

URL: <https://www.wjgnet.com/1007-9327/full/v26/i18/2194.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v26.i18.2194>

INTRODUCTION

Together with ulcerative colitis, Crohn's disease (CD) represents the most common and clinically relevant inflammatory bowel disease (IBD)^[1,2]. While it is generally accepted that the pathogenesis of the disease is multifactorial and involves an inappropriate activation of the mucosal immune system, the precise contribution of individual environmental factors and genetic traits remains elusive^[1-3]. Mutations in the *NOD2* gene represent the best-characterized genetic association of CD^[4-6]. Nucleotide-binding oligomerization domain 2 (*NOD2*) belongs to the pattern recognition receptor (PRR) family and acts as an intracellular sensor for peptidoglycan^[7,8] and its fragment muramyl dipeptide^[9,10]. Downstream of *NOD2*, the transcription factor NF-κB plays a key role in the transduction of receptor-generated signals^[11].

C-type lectin domain (CLEC) receptors comprise a large family of carbohydrate-binding proteins^[12]. Various CLEC family receptors are considered to exert functions as PRR since they recognize pathogen-associated molecules and may induce intracellular signaling pathways that regulate inflammatory processes. CLEC proteins are crucially involved in the immune response to fungal pathogens, but have also been implicated in anti-bacterial, anti-viral and anti-parasitic defense mechanisms^[13,14]. Despite their functional similarities to *NOD2*, CLEC proteins have not been systematically studied in the context of IBD yet. Interestingly, a single nucleotide polymorphism (SNP) in the *CLEC7A* (*DECTIN-1*) gene, rs2078178, has been reported to be strongly linked to a severe form of ulcerative colitis, and this association was even stronger for the two-marker haplotype rs2078178 to rs16910631^[15]. For another CLEC gene, *CLEC5A*, we recently observed a CD-associated expression pattern with higher transcript levels in patient-derived peripheral blood mononuclear cells than in corresponding controls. Furthermore, *CLEC5A* showed a *NOD2*-dependent expression profile, supporting the hypothesis that both proteins may act in a regulatory network with a pathophysiological role in CD^[16]. Given that defective bacterial clearance may contribute to the pathogenesis of CD^[17,18], it is important to note that *CLEC5A* has also been suggested to be essentially involved in innate immunity through neutrophil trap formation and secretion of different proinflammatory cytokines after stimulation with *Listeria monocytogenes*^[19]. Interestingly, the SNP rs1285933 in *CLEC5A* is associated with dengue severity^[20], and

CLEC5A has been shown to be critical for dengue-virus-induced lethal disease^[21].

Here, we have addressed the question if the SNPs rs2078178 and rs16910631 in *CLEC7A* and rs1285933 in *CLEC5A* are associated with CD and have analyzed effects of rs1285933 at the level of gene expression. For comparison and a positive control, the known disease-associated SNPs rs2066844 (SNP8), rs2066845 (SNP12) and rs2066847 (SNP13)^[5,6] in *NOD2* were included into the investigations as well.

MATERIALS AND METHODS

Patients

From October 2015 until June 2017, 175 patients (102 females and 73 males; mean age 43.1 ± 14.7 years) with CD from the Department of Gastroenterology of Rostock University Medical Center (Rostock, Germany) were included in the study. This cohort of CD patients represents an extension of a cohort that we have previously characterized regarding relationships between mutations in the *NOD2* gene, the disease phenotype and anti-tumor necrosis factor- α trough levels^[22].

The diagnosis of CD was based on clinical, endoscopic, histological and radiological findings of the patients. The following clinical data were collected: Age, sex, age at diagnosis, duration of the disease, disease location, disease behavior, disease activity (assessed by the Crohn's disease activity index^[23] and the Harvey-Bradshaw index^[24]), disease-specific medications, and previous history of surgery (*i.e.*, colectomy). CD was stratified *via* the Montreal classification^[25]. Unrelated and healthy subjects from Germany ($n = 157$; 101 females and 56 males; mean age 25.3 ± 5.7 years) served as controls. The study was approved by the Local Ethics Board of the University of Rostock (A-2017-0137). We obtained written informed consent from all participants prior to their enrollment.

DNA extraction

EDTA whole-blood samples were subjected to DNA extraction employing the QIAamp DNA blood mini kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany).

Genotyping

Genotyping was performed using TaqMan™ SNP Genotyping Allelic Discrimination Assays with VIC- and FAM-labeled probes (Thermo Fisher Scientific, Karlsruhe, Germany) for rs1285933 (*CLEC5A*, Assay-ID: C__9506735_10), rs2078178 (*CLEC7A*; Assay-ID: C__1932439_10), rs16910631 (*CLEC7A*; Assay-ID: C__33748498_10), rs2066844 (*NOD2*, SNP8, Assay-ID: C__11717468_20), rs2066845 (*NOD2*; SNP12, Assay-ID: C__11717466_20), and rs2066847 (*NOD2*, Assay-ID: SNP13 C__60383785_10). PCR was carried out in 96-well plates, employing a ViiA 7 sequence detection system (Thermo Fisher Scientific). Thermal cycling conditions were: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C/1 min at 60 °C. After PCR, fluorescence was detected and analyzed using TaqManGenotyper software version 1.3. Alternatively, *NOD2* genotypes were determined by Sanger sequencing as described before^[22].

In vitro studies with peripheral blood mononuclear cells

In this study, previous data from our laboratory were re-evaluated with respect to the rs1285933 genotype^[16]. Briefly, peripheral blood mononuclear cells (PBMC) had been isolated from EDTA venous blood, cultured and treated with lipopolysaccharide (1 µg/mL; Sigma-Aldrich, Deisenhofen, Germany) for 6 h. Afterwards, RNA was isolated, reversely transcribed into cDNA and subjected to real-time PCR employing standard procedures and a ViiA 7 sequence detection system. The following human-specific TaqMan™ gene expression assays with fluorescently labeled MGB probes were used to quantify target cDNA levels: Hs04398399_m1 (*CLEC5A*), Hs01099660_g1 (*CXCL5*), and Hs99999905_m1 (*GAPDH*). PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C/1 min at 60 °C.

Statistical analysis

The data were stored and analyzed employing IBM SPSS Statistics 25.0 (International Business Machines Corporation, Armonk, New York, United States). Differences between patients and controls were assessed for distributions (genotype, allele and sex) using the χ^2 test or Fisher's exact test, and for means using the *t*-test for independent samples (age, gene expression data), respectively. Pairwise statistical interaction between SNPs in a linear model was studied employing ANOVA. The Hardy-Weinberg equilibrium was assessed using the χ^2 test with 1 degree of freedom. False discovery rates were controlled by using the Benjamini-Hochberg correction.

Values of $P < 0.05$ were considered statistically significant.

RESULTS

SNP genotyping was performed on DNA samples from 175 patients with CD and 157 healthy controls. Both study groups are comparable for distribution of sex ($P = 0.310$), while patients with CD were older than healthy volunteers who served as controls (43.1 ± 14.7 vs 25.3 ± 5.7 years; $P < 0.0001$). In the context of this study, this age difference was considered acceptable. For the controls, the distribution of all individual SNP genotypes was in accordance with the Hardy-Weinberg equilibrium.

To study associations of CD with SNP genotypes or allele frequencies, four genetic models (genotype, dominant, recessive, or allelic models) were employed (Table 1). As expected, significant associations with CD were found for SNPs in *NOD2*, specifically rs2066844 (SNP8; genotype $P = 0.0498$, dominant P value = 0.0219, allelic P value = 0.0368) and rs2066847 (SNP13; allelic P value = 0.0474). Intriguingly, the genotype AA of rs1285933 in *CLEC5A* was also potentially associated with the disease (recessive model; $P = 0.0523$). The corresponding odds ratios (ORs) are shown in Table 2. For *NOD2*, the odds of having CD might triple in the presence of the risk allele T (rs2066844: OR = 3.29), and double with allele CC (rs2066847: OR = 2.31). Increased ORs are detectable for *CLEC5A*, too. Genotype AA almost doubles the odds of CD (OR = 1.90). Carrying the risk allele A increases the odds of CD by 39% (OR = 1.39), whereas allele G displays a protective effect (OR = 0.72). We could not detect significant associations between CD and the two SNPs in *CLEC7A* (rs2078178, rs16910631) and also not for rs2066845 (SNP12) in *NOD2* (Table 1). The latter finding might be explained by the rare occurrence of the risk allele C in our cohorts of small size.

We next compared patients with different genotypes of rs1285933 in *CLEC5A* (AA, AG and GG, respectively) regarding their clinical characteristics, employing the following parameters: Age, age at diagnosis, duration of the disease, disease location and behavior according to Montreal classification, Crohn's disease activity index and Harvey-Bradshaw index, history of surgical treatment and treatment with drugs (including antibodies such as tumor necrosis factor- α inhibitors). There were no statistically significant differences between the three genotypes (data not shown).

To study potential functional effects of the rs1285933 polymorphism, we re-evaluated previously published gene expression data from our laboratory. In these studies, PBMC from CD patients and controls had been employed to measure the mRNA expression of a pre-selected set of genes^[16]. Using a combined data set from 16 CD patients and 6 healthy controls, we observed no genotype-dependent differences of *CLEC5A* gene expression (Figure 1A). On the other hand, we found that the genotype GG, compared to AG, was associated with significantly lower mRNA levels of the proinflammatory chemokine *CXCL5* (Figure 1B, please note that ΔCt values and expression levels show an inverse and logarithmic relationship that follows the function $2^{-(\Delta\text{Ct})}$).

Located on different chromosomes, the disease-associated SNP of *CLEC5A* is not correlating with disease-associated SNPs of *NOD2* (data not shown). Furthermore, the pairwise contributions to the disease phenotype of the *CLEC5A* SNP and the other SNPs are independent from each other (Table 3).

DISCUSSION

To the best of our knowledge, the results of this study suggest for the first time a potential association of SNP rs1285933 with CD. However, our findings need to be interpreted cautiously since they are based on a relatively small number of patients from a single center.

Given that the SNP is located within the *CLEC5A* gene, our data implicate a PRR beyond *NOD2* into the pathogenesis of the disease. The mechanisms that underlie the effect of the *CLEC5A* polymorphism need to be further elucidated. To this end, reinvestigating data from our past work^[16] we can report a *trans* effect of rs1285933 on the expression of the chemokine *CXCL5* in PBMC, but not of *CLEC5A* itself. These data suggest that *CLEC5A* might be functionally affected, *e.g.*, with respect to its ability of ligand binding or downstream signaling. In accordance with this conclusion, SNP rs1285933 has also been suggested to modulate signaling pathways after interactions between the dengue virus and *CLEC5A* receptors^[20]. Other disease associations of SNP rs1285933 have not been reported yet. In a population of Taiwanese children, neither rs1285933 nor other polymorphisms of *CLEC5A* were

Table 1 Genotype and allele frequencies of single nucleotide polymorphisms in the genes *CLEC5A*, *CLEC7A* and *NOD2* in Crohn's disease patients and controls

Gene	SNP	Genotype	Cases (n = 175)	Controls (n = 157)	Allele ¹	Cases (alleles)	Controls (alleles)	Genotype P value ²	Dominant P value ^{2,3}	Recessive P value ^{2,3}	Allelic P value ²
<i>CLEC5A</i>	rs1285933	GG	35	36	G, A	144, 206	155, 159	0.1093 (0.0285)	0.9727 (0.5921)	0.0523 (0.0091)	0.0900 (0.0352)
		GA	74	83							
		AA	66	38							
<i>CLEC7A</i>	rs2078178	GG	104	100	G, A	274, 76	251, 63	1.0000 (0.5713)	0.9033 (0.4320)	0.8344 (0.7618)	0.9107 (0.6335)
		AG	66	51							
		AA	5	6							
<i>CLEC7A</i>	rs16910631	CC	153	139	C, T	327, 23	294, 20	0.8078 (0.7024)	0.9056 (0.8662)	0.9269 (0.6045)	1.0000 (1.0000)
		CT	21	16							
		TT	1	2							
<i>NOD2</i>	rs2066844 (SNP8)	CC	146	148	C, T	319, 31	305, 9	0.0498 (0.0065)	0.0219 (0.0019)	0.9583 (0.5000)	0.0368 (0.0016)
		CT	27	9							
		TT	2	0							
<i>NOD2</i>	rs2066845 (SNP12)	GG	163	149	G, C	338, 12	306, 8	0.8481 (0.6453)	0.8481 (0.6453)	NA	0.7874 (0.6505)
		GC	12	8							
		CC	0	0							
<i>NOD2</i>	rs2066847 (SNP13)	C-C	147	143	C, CC	316, 34	300, 14	0.0923 (0.0321)	0.1569 (0.0682)	0.1025 (0.0312)	0.0474 (0.0103)
		C-CC	22	14							
		CC-CC	6	0							

¹Italic: Minor allele according to database <https://www.ncbi.nlm.nih.gov/snp/>.²Numbers in brackets refer to the P value prior to Benjamini-Hochberg correction (23 tests); significant differences ($P < 0.05$) are indicated in bold.³Refers to the minor allele. SNP: Single nucleotide polymorphism; NA: Not applicable (due to the absence of CC genotype).

associated with susceptibility to Kawasaki disease, coronary artery lesion formation, and intravenous immunoglobulin treatment response^[26].

Of note, *CLEC5A* is embedded into an intronic region of another gene, *MGAM*, and the two transcripts are known to correlate^[27], so that the effect of SNP rs1285933 is not necessarily exclusively related to the C-type lectin domain family member. Interestingly, decreased maltase activities in the small bowel mucosa are common in children with CD^[28], and although this is of course no evidence for a genetic association, the role of *MGAM* in the context of IBD may deserve further attention as well.

We also evaluated possible associations of SNP rs1285933 with different clinical characteristics of our CD patients, including disease location, disease behavior and treatment history, but did not obtain significant results. Given that such effects have been reported for *NOD2* variants^[29-32], the studies are nevertheless worth to be continued in larger cohorts of patients. To this end, we conclude that the principal effect of SNP rs1285933 is modulation of CD susceptibility through a different molecular pathway than *NOD2*.

PRRs are key regulators of innate immune responses and inflammatory processes^[13,14]. For a prominent member of this family, *NOD2*, a role in the pathogenesis of CD is clearly established^[4-6]. Our results suggest an association of a polymorphism in another PRR, rs1285933 in *CLEC5A*, but not of rs2078178 and rs16910631 in *CLEC7A*, with CD. A systematic analysis of PRR functions in the context of CD might reveal novel pathomechanistic insights and help to identify new targets for diagnostic and therapy.

Table 2 Odds ratios of genotypes and alleles of single nucleotide polymorphisms in the genes *CLEC5A* and *NOD2*

Gene	SNP	Genotype/allele	Odds ratio	95%CI ¹	P value ¹
<i>CLEC5A</i>	rs1285933	AA	1.90	1.18-3.05	0.009
		GG	0.84	0.50-1.42	0.516
		AG	0.65	0.42-1.01	0.054
		A	1.39	1.03-1.90	0.034
		G	0.72	0.53-0.97	0.034
<i>NOD2</i>	rs2066844 (SNP8)	TT	NA		
		CC	0.31	0.14-0.67	0.003
		CT	3.00	1.36-6.60	0.006
		T	3.29	1.54-7.03	0.002
		C	0.30	0.14-0.65	0.002
<i>NOD2</i>	rs2066847 (SNP13)	CC-CC	NA		
		C-C	0.51	0.26-1.02	0.056
		C-CC	1.47	0.72-2.98	0.287
		C	0.43	0.23-0.82	0.011
		CC	2.31	1.21-4.38	0.011

¹Unadjusted for multiple testing. SNP: Single nucleotide polymorphism; NA: Not applicable (missing in controls); CI: Confidence interval.

Table 3 Pairwise statistical interaction between single nucleotide polymorphisms in a linear model¹

SNP	<i>CLEC5A</i>	<i>CLEC7A</i>	<i>NOD2</i>			
	rs1285933	rs2078178	rs16910631	rs2066844	rs2066845	rs2066847
rs1285933	NA	0.6490	0.7409	0.5266	0.6875	0.2813
rs2078178	0.6490	NA	0.1036	0.8573	0.4040	0.3718
rs16910631	0.7409	0.1036	NA	0.8980	0.6698	0.9270
rs2066844	0.5266	0.8573	0.8980	NA	2.8248e-07	0.9664
rs2066845	0.6875	0.4040	0.6698	2.8248e-07	NA	0.7399
rs2066847	0.2813	0.3718	0.9270	0.9664	0.7399	NA

¹Disease—single nucleotide polymorphism (SNP) A + SNP B + SNP A: SNP B. The uncorrected *P* values for the last term of an ANOVA are presented in the table for all 30 interactions. The only significance was for two chromosomally neighboring SNPs within *NOD2*. SNP: Single nucleotide polymorphism; NA: Not applicable.

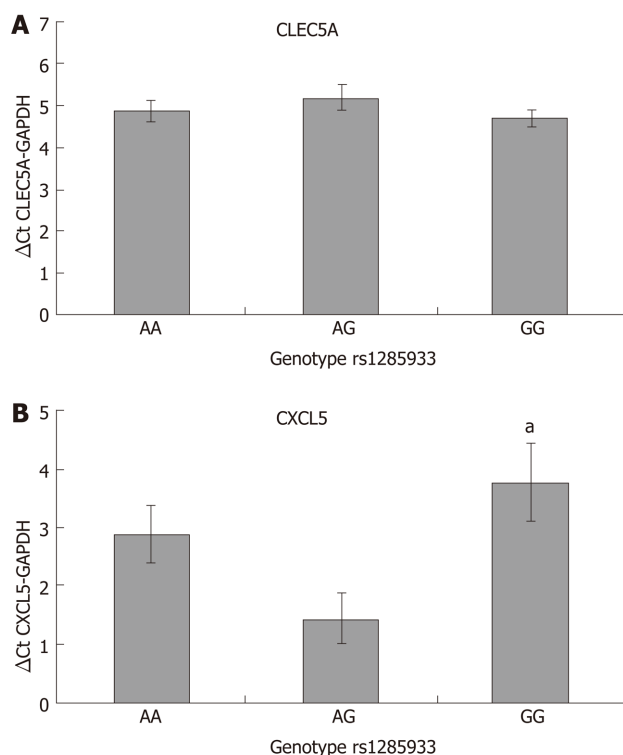


Figure 1 Effects of the rs1285933 genotype on *CLEC5A* and *CXCL5* gene expression. Peripheral blood mononuclear cells were isolated from individuals with genotype AA ($n = 8$), GG ($n = 5$), and AG ($n = 9$), cultured and treated with lipopolysaccharide (1 $\mu\text{g/mL}$) for 6 h. Subsequently, the mRNA expression of the indicated genes and the house-keeping control *GAPDH* was analyzed by real-time PCR. Data are presented as averaged ΔCt values \pm standard error of mean. ^a $P < 0.05$ vs genotype GG.

ARTICLE HIGHLIGHTS

Research background

Crohn's disease (CD) is characterized by a multifactorial etiology and a significant impact of genetic traits. While *NOD2* mutations represent well established risk factors of CD, the role of other genes is incompletely understood.

Research motivation

A better knowledge of the molecular basis of CD is considered as an essential prerequisite for a further improvement of diagnostics and therapy.

Research objectives

Previous studies from our laboratory have pointed to a possible link between CD and the expression of pattern recognition receptors of the C-type lectin domain family (specifically, *CLEC5A*) in peripheral blood mononuclear cells (PBMC). This observation prompted us to ask if single nucleotide polymorphisms in the genes *CLEC5A* and *CLEC7A* might be associated with the disease.

Research methods

DNA samples from patients with CD and healthy donors were subjected to the analysis of single nucleotide polymorphisms in the genes *CLEC5A*, *CLEC7A* and *NOD2*. For studies on gene expression, PBMC from subgroups of both cohorts were employed. Molecular findings were correlated with clinical characteristics of the patients.

Research results

For genotype AA of rs1285933 in *CLEC5A*, a potential association with CD and an increased odds ratio were detected. As expected, risk variants of *NOD2* were associated with an increased occurrence of CD as well. Polymorphisms of rs1285933 correlated with *CXCL5* gene expression but had no effect on *CLEC5A* expression in PBMC.

Research conclusions

SNP rs1285933 in *CLEC5A* may represent a novel genetic association of CD. The finding, however, needs to be reproduced in multicenter studies with larger numbers of CD patients.

Research perspectives

Pattern recognition receptors of the C-type lectin domain family deserve further attention

regarding their potential role in the pathogenesis of CD and their relevance as diagnostic markers and therapeutic targets.

ACKNOWLEDGEMENTS

We thank Mrs. Katja Bergmann for expert technical assistance.

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