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Disease duration and age influence CARD15 expression in Crohn's disease

Czas trwania choroby i wiek wpływają na ekspresję CARD15 w chorobie Leśniowskiego-Crohna

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

One of the susceptibility genes in Crohn's disease (CD) is CARD15. Our study examined the relationship between peripheral CARD15 expression and phenotype and duration of CD, treatment methods and inflammatory indices. Sixty patients with CD and 30 healthy volunteers as controls were enrolled in the study. Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) with E.Z.N.A. Total RNA Kit (Omega Bio-tek) then quantitative real-time PCR was performed on the ABI Prism 7900 HT Real-Time PCR System. CARD15 gene expression in PBMCs in CD was significantly higher than in the control group. The highest level of gene expression was found in CD patients in the fourth decade of life. The mRNA level of the CARD15 gene was higher in patients with disease duration between 12 and 60 months. A positive correlation was found between erythrocyte sedimentation rate (ESR) and gene expression level. Gene expression increased with increasing level of C-reactive protein and ESR, but it was not statistically significant. CARD15 expression significantly decreased in CD patients treated with anti-TNF α agents compared to azathioprine or steroid treatment groups. Expression of the CARD15 gene in Crohn's disease is higher than in healthy individuals. Disease duration and age of patients seem to be the most important factors influencing CARD15 expression.

Key words: CARD15 • inflammatory bowel disease • Crohn's disease

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory conditions affecting the gastrointestinal tract, encompassing ulcerative colitis (UC), Crohn's disease (CD) and undetermined colitis. IBD most often affect young people and may cause a significant decrease in quality of life [7,12]. The etiopathogenesis of IBD is complex and remains unclear, but it seems that genetic, environmental, and immunological factors play an important role in its etiology. A strong genetic contribution of Crohn's disease has been suggested by twins studies and familial association [7]. A genome-wide linkage analysis identified a susceptibility gene for CD on chromosome 16q12, originally reported as *NOD2* and recently renamed *CARD15* [11]. *CARD15* is well known as a susceptibility gene in CD. Additionally, it is considered as a disease-modifier gene for CD. It influences disease location (ileal disease), disease behavior (structuring disease) and early age of onset [13].

The *CARD15* protein (1040 amino acids) contains two N-terminal caspase activation and recruitment domains (CARDs), a centrally located nucleotide-binding oligomerization domain (NOD) and leucine-rich repeats (LRR) at the C terminus. The LRR domains are important for bacterial binding, recognizing muramyl dipeptide (MDP) derived from bacterial peptidoglycan (PGN), a major cell wall component of most Gram-positive and Gram-negative bacteria. Binding of MDP to the LRR domains leads to nuclear factor κ B (NF- κ B) activation and induces NF- κ B-dependent immune response gene expression under normal circumstances [9,11]. NF- κ B is a key signaling molecule and has been shown elevated in CD tissues [10]. More than 60 CD-associated polymorphisms have been identified in the *CARD15* gene, but the most common three mutations (R702W, G908R, and 1007fs) are associated with susceptibility to CD [2,3,4]. Thus *CARD15* seems to be important for the pathogenesis of CD.

The aim of the study was to examine the relationship between peripheral *CARD15* gene expression and treatment methods, disease phenotype, disease duration, as well as inflammatory parameters in Crohn's disease patients.

MATERIAL AND METHODS

Patient population

Sixty patients (30 women and 30 men, aged 21–65, mean age 39.2) hospitalized in the Department of Gastroenterology and Hepatology, Wrocław Medical University with the diagnosis of Crohn's disease and 30 healthy volunteers as a control group were enrolled in the study.

The level of gene expression was assessed in peripheral mononuclear cells in all patients and the control group.

Analysis between gene expression in CD patients and the following parameters was performed:

1. age and gender,
2. duration of disease,
3. administered treatment (steroids (n=24), azathioprine (n=28), anti-TNF therapy (n=11)),
4. markers of inflammation (erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet count (PLT) and fibrinogen),
5. disease location, presence of anemia, fistulas, strictures and extraintestinal symptoms.

The study was conducted in accordance with the Helsinki Convention and approved by the local Ethics Committee of Wrocław Medical University. Written informed consent was obtained from each patient.

RNA isolation and cDNA synthesis

Total RNA was isolated from PBMCs with E.Z.N.A. Total RNA Kit (Omega Bio-tek) according to the manufacturer's protocol. First strand cDNA was synthesized from 5 μ l of DNA-free RNA of each sample using TaqMan Reverse Transcription Reagents (Roche) with oligo(dT)₂₀ primer. The 20 μ l reaction mixtures were incubated in an Applied Biosystems Thermocycler for 10 min at 25°C, 30 min at 48°C. The cDNAs were then used as templates for real-time PCR, the most sensitive and reliable method for detection and quantitation of nucleic acid levels.

Real-time PCR

Quantitative real-time PCR was performed on the ABI Prism 7900 HT Real-Time PCR System. All PCRs were performed using 5 μ l cDNA per reaction in triplicates of 25 μ l volume. Universal PCR Master Mix with UNG (uracil N-glycosylase) was obtained from Applied Biosystems; it included all reagents including Taq-polymerase apart from specific primers and probes. All TaqMan gene expression assays were obtained from Applied Biosystems, *CARD15* (Hs00223394_m1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1). The reaction mixtures were incubated in a 96-well plate at 95°C for 10 min, following 40 cycles of 95°C for 15 s and 60°C for 1 min.

Dilution experiments were performed to ensure similar efficiency of the PCRs, and standard curves were calculated referring the threshold cycle to the log of each cDNA dilution step (Ct, the PCR cycle at which a specific fluorescence becomes detectable). The mRNA level of the housekeeping gene (*GAPDH*) was used as an endogenous control, and gene-specific mRNA expression was normalized against *GAPDH* expression. All data were analyzed by relative quantification $\Delta\Delta C_t$ methods. The Ct values from three separate cDNA runs were averaged; if the standard deviation of the Ct values was above 0.16, the sample was not used in the analysis.

Statistical methods: Mann-Whitney test was used.

RESULTS

1. Gene *CARD15* expression in PBMCs in patients was significantly higher than in the control group (Figure 1) ($p < 0.05$).

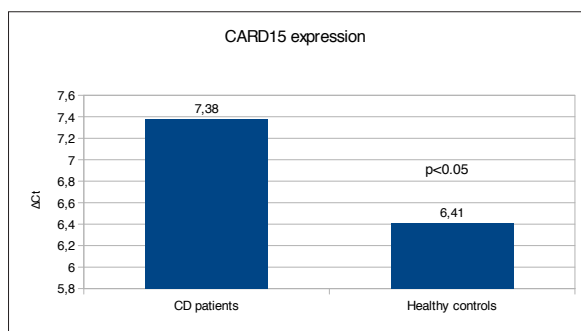


Fig. 1. Level of *CARD15* gene expression in CD patients compared with control group

2. We found a similar ΔCt value in male and female CD patients.

3. The highest level of gene expression was found in CD patients in the fourth decade of life, compared to the mean gene expression in CD patients ($p < 0.05$) (Figure 2a).

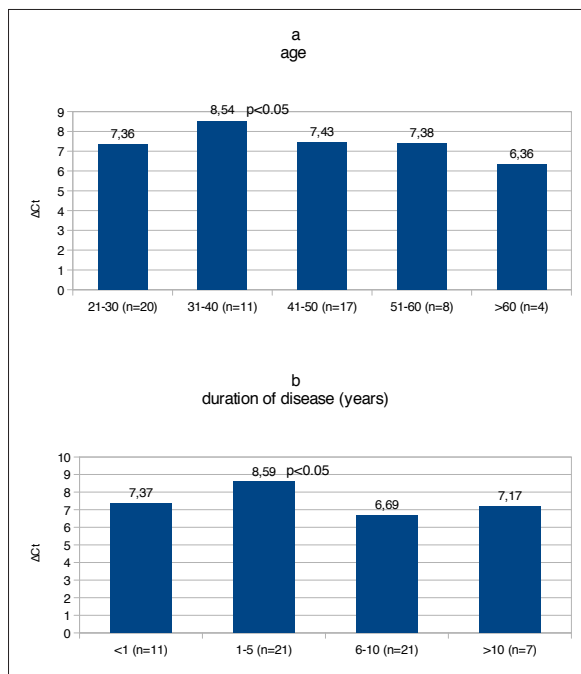


Fig. 2. Level of *CARD15* gene expression in CD patients depending on analyzed parameters

4. A higher mRNA level of the *CARD15* gene in patients with disease duration between 12 and 60 months (Fig-

ure 2b) compared to mean gene expression in CD patients ($p < 0.05$) was observed.

5. A positive correlation was found between CRP and ESR and gene expression level (Figure 2c, 2d) but it was not statistically significant. We did not find an association between ΔCt value and PLT or fibrinogen.

6. This study showed that *CARD15* expression significantly decreased in CD patients treated with anti-tumor necrosis factor alpha (anti-TNF α) agents compare to azathioprine or steroid treatment groups (Figure 3) ($p < 0.05$).

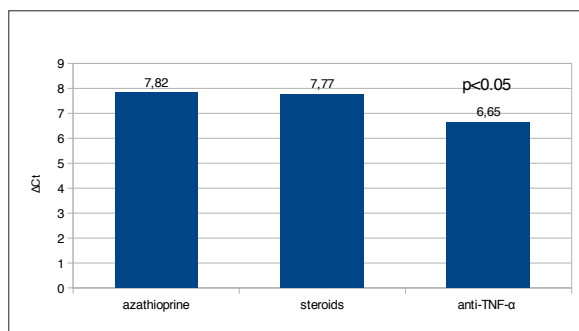


Fig. 3. Level of *CARD15* gene expression in CD patients treated with azathioprine, steroid and anti-TNF- α agents

7. Our study did not reveal a correlation between level of the *CARD15* gene and localization of inflammatory changes in the gastrointestinal tract, presence of extraintestinal symptoms, anemia, fistulas or operation.

DISCUSSION

We found that gene *CARD15* expression in Crohn's disease in peripheral blood mononuclear cells is higher when compared to healthy individuals. Additionally, we found that age, disease duration and administered treatment may influence *CARD15* expression in Crohn's disease patients.

CARD15 is an intracellular protein expressed in monocytes, macrophages, neutrophils and dendritic cells, as well as in Paneth cells in the small intestine. Its expression in enterocytes is low under normal but increased under inflammatory conditions [2,5,8,14].

In our study there was a positive but not significant correlation between peripheral expression of the studied gene and the classical marker of inflammation ESR. Additionally, an association between *CARD15* expression and CRP, currently the most reliable inflammation parameter used in clinical practice, was observed.

As reviewed by Li and Kuemmerle, beside innate immune system components and autophagy, mutations in NOD2/*CARD15* play a significant role in the pathogenesis of fibrosis in CD [6]. In our study, location of the inflamma-



tory changes in the gastrointestinal tract, presence of fistula, strictures, extraintestinal symptoms and anemia did not influence gene expression. Thus, it seems that inflammatory activity of Crohn's disease may not be considered as a major factor regulating peripheral expression of the *CARD15* gene. Bhullar et al. reported that CD patients with *NOD2/CARD15* mutations have a more aggressive course of the disease and require multiple surgical interventions [1].

NOD2/CARD15 mutations do not influence the response to treatment with systemic steroids, azathioprine and infliximab [15]. In our study patients treated with biologic therapy (anti-TNF- α agents) had lower gene expression when compared to patients treated with azathioprine and steroids. Possible remodeling of gene expression by anti-TNF- α agents still needs further studies.

We found similar Δ Ct values in male and female CD patients, suggesting that gene expression level is not a sex-related trait.

Elucidation of the mechanisms of Crohn's disease and the role of the *CARD15* gene requires further analysis of *CARD15* gene expression in tissues, particularly in the colon, the main site of interaction with bacterial microflora.

In summary, expression of the *CARD15* gene in Crohn's disease is higher than in healthy individuals, and disease duration and age of the patient seem to be the most important factors influencing *CARD15* expression.

A limitation of the study was the small number of patients in the studied group. Further investigations are needed to elucidate the influence of *CARD15* expression on clinical history of Crohn's disease.

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The authors have no potential conflicts of interest to declare.