STATISTICAL INTERACTIONS OF POLYMORPHISMS ASSOCIATED WITH CROHN'S DISEASE IN THE HUNGARIAN POPULATION

PhD thesis

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Pécs
2014

INTRODUCTION

Two main clinical presentations of inflammatory bowel disease (IBD) are Crohn's disease (CD) and ulcerative colitis (UC). IBD is now widely believed to originate from an uncontrolled mucosal immunity of the gastrointestinal tract; however the exact process of CD pathogenesis is still unknown. Twin and family studies have reported that besides environmental factors genetic susceptibility is also essential in IBD development. Up to date, many novel candidate genes have been found to confer increased risk for the disease, some loci seem to be specific to CD or UC, others have been reported to confer susceptibility to IBD overall; the most replicated loci are *CARD15*, IBD5, *IL23R* and *ATG16L1*. Despite the success of genome wide association studies (GWAS) in identifying significantly associated loci, they are estimated to account for only 25% of predicted heritability. The numerous new genetic variants reported by these studies are usually common in the population but have only slight effect on disease risk, only a few polymorphisms with stronger effect have been described yet. The rising number of CD candidate genes gives us the possibility to evaluate gene-gene interactions among susceptibility genes. Playing a role in the biomolecular mechanisms, these interactions, or epistasis are ubiquitous features of the genetic architecture of common human diseases; their existence have been proved by several studies. By the original definition of epistasis one gene masks the effects of another gene. The definition on the population level describes epistasis as deviations from additivity in a statistical model, and has rather statistical features instead of dealing with biological functions. Since gene-gene interactions cannot only enhance but also weaken the individual gene effects, which can explain the lack of replication of single-locus results, complex gene-gene interactions may be considered more important than independent effects of single susceptibility genes. Nevertheless the identification of high-risk gene combinations might help to improve disease prediction even if the exact biological reasons are missed.

Caspase activation recruitment domain containing protein 15 *(CARD15)*

Receptors recognizing microbe associated molecular patterns are central to innate immunity (pattern recognition receptors, PRR). The nucleotide-binding oligimerization domain containing 2 (NOD2) protein, which belongs to the long family of Nod-like receptors (NLR) shows univoque association to CD pathogenesis. The ligand of this cytoplasmic protein is the muramyl-dipeptid (MDP), the cell wall component found in wide-range of Gram+ and Gram- bacteria; that is why NOD2 can be activated by numerous bacterial invadors, like *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus*.

The intacellular receptor NOD2 functions as an "emergency sensor" for true bacterial threats that can gain access to the cytoplasm. Upon activation NOD2 associates with the protein kinase RICK/Rip2 (CARD3) and CARD9, leading to activation of nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pathways and subsequent induction of pro-inflammatory cytokines (like TNFα, IL6 és IL1β). The receptor is mainly expressed in leukocytes, endothelial cells and fibroblast; but can be found in the intestinal epithelium and in the Paneth cells of the ileum as well.

NOD2 is encoded by *CARD15* gene at 16q12 chromosome region. Of the more than 60 reported sequence variants of the NOD2 protein, three main variants, R702W (rs2066844), G908R (rs2066845), and 1007fs (rs2066847) have the strongest CD association. The 1007fs frameshift mutation, which shows the strongest association with CD, encodes a truncated protein with impaired membrane binding. The *CARD15* variants are common in Caucasian patients, however their prevalence is population dependent. Individuals with one of the three major disease-associated NOD2 alleles have a 2- to 4-fold increased risk of developing Crohn's disease, while homozygous or compound heterozygous carriers have a 20- to 40-fold increase in risk, the effect is CD specific. In the Hungarian population Lakatos et al. reported significant association of R702W and 1007fs with CD, the third variant has neutral effect. The disturbed function of NOD2 probably leads to the imbalance of the intestinal immune homeostatis, the role of NOD2 variants in this processis is not completely understood. They influence the antimicrobial peptide production of Paneth cells, the insufficient activity of NOD2 leads to decreased defensin secretion and subsequent invasion of bacteria into the mucosa.

Autophagy-related 16-like 1 (*ATG16L1*)

Autophagy is a fundamental cytoplasmic homeostasis process enabling individual cells to clean up, in a highly regulated fashion, their own cytoplasm by sequestering portions of the cytoplasm and degrading the captured constituents. It plays crucial roles in developmental, differentiation processes and in the immune system for elimination of intracellular microbes, presentation of endogenous antigens via major histocompatibility complex class II, shaping B- and T-cell function, and defining central tolerance. Multiple autophagy-related (ATG) proteins are required for the precise execution of the autophagic process; one of them is the ATG16L1, which has modulatory role in the mechanism. The large 350 kDa complex of ATG16L1 and ATG5-ATG12 is responsible for the membrane localisation of the autophagic machinery and formation of the autophagosome. The gene

encoding ATG16L1 protein is localized in the 2q37.1 chromosome region and contains 19 exons. In a European GWAS the coding variant T300A (rs2241880) within the *ATG16L1* gene was reported to be highly associated with CD, and to carry the whole disease risk exerted by this locus. The association of *ATG16L1* gene and CD was replicated in numerous studies; the T300A was proved to be risk variant in the Hungarian CD population, as well. ATG16L1 is mainly expressed in the colon, small bowel, intestinal epithelial cells, leukocytes, and spleen. In the Paneth cells of the distal ileum it plays role in the exocytosis of secretion granules containing antimicrobial peptides, like defensin. These cells show aberrant cell morphology in the tissue biopsy of CD patients with *ATG16L1* homozygote genotype.

Interleukin-23 receptor (*IL23R*)

The intestinal tissue damage of Crohn's disease patients is mainly caused by the excessive activation of the adaptive immunity. In CD, Th1 lymphocytes, characterized by elevated production

of IL2, IL12 and IFNγ, are predominant. Besides Th1 cells the CD4+ and CD25- Th17 lymphocyte population has a central role in he develpoment of CD too. The main characteristic of these cells is the production of the proinflammatory cytokine IL17. Th17 differentiation of naive Th0 cells is induced by the coexpression of IL23 and TGFβ whose role in determining the balance between regulatory T (Treg) lymphocytes and proinflammatory Th17 lymphocytes is essential. In the absence of IL23, TGFβ induces Treg differentiation; while IL23 decreases the pool of FoxP3+ cells in the intestine and enhances the IL17 secretion of Th17 cells promoting the development of mucosal inflammation. IL-23, mainly expressed by activated macrophages and dendritic cells, is a heterodimeric cytokine consisting of two subunits: the IL23-specific p19 and p40, which is also a component of IL12. IL17A secreted by Th17 lymphocytes induces the subsequent production of inflammatory mediators and activation of neutrophils via its receptors expressed on leukocytes, fibroblasts, epithelial and endothelial cells. The imbalance of immunregulation leads to autoimmune and inflammatory diseases. The ileal dendritic cells of CD patients and mice with T-cell mediated experimental colitis secrete increased level of IL23R, which confirms the pivotal role of IL23/IL17 axis in the development of chronic inflammatory processes like Crohn's disease. The heterodimer receptor of IL23 (IL23R), mainly expressed on activated myeloid cells (macrophages, dendritic cells) and T-lymphocytes shares one subunit with IL12R (IL12Rβ) the other one is IL23R specific. In the area between genes encoding IL23R and IL12Rβ and in the *IL23R* gene itself Duerr et al. identified 10 polymorphisms (rs11209026, rs1004819,

rs7517847, rs10489629, rs2201841, rs11465804, rs1343151, rs10889677, rs11209032, rs1495965) associated with CD risk. The relationship between disease depevelopment and the IL23R SNPs was verified by numerous international studies focusing mostly on the rare missense R381Q variant with relatively strong protective effect. The association between reduced CD risk and R381Q was confirmed in a Hungarian study as well; while the rs10889677 in the 3'UTR region of *IL23R* and the intronic rs2201841 were found to confer risk for Crohn's disease.

Cytotoxic T lymphocyte antigen-4 (*CTLA4*)

The *CTLA4* gene is mainly expressed in activated T cells, and plays role in the downregulation of T-cell activation and self-tolerance. CTLA4, a member of the immunoglobulin subfamily, functions as a T-cell receptor binding to B7-1 (CD80) and B7-2 (CD86) against another competitive receptor, CD28, which operates as an upregulator of T-cell activation. Ligation of CTLA4 transmits inhibitory signals towards activated T-cells. The CD28 and CTLA4 proteins play role in the negative selection of thymocytes during their maturation in the thymus: the CD28 kostimulation induces the clonal deletion of autoreactive CD4+ T cells, while the signal via CTLA4 enhances the survival of thymocytes. The majority of FoxP3+ Treg cells constitutively express high levels of CTLA4 as well. In vivo blockade of CTLA4 in mice leads to the spontaneous development of organ-specific autoimmune disorders, like IBD and diabetes. To date the role of the *CTLA4* gene +49 A/G variant remained uncertain in IBD, the available results on +49 A/G in IBD and Crohn's disease are still conflicting. The +49 GG genotype was described to be associated with CD susceptibility, but only in the subgroup of Japanese patients with fistula. Contrarily, in the study of Ben Alaya et al. both the +49 A allele and the AA genotype were found to confer risk for CD development. No association was found either in Dutch IBD or in Chinese and Iranian UC patients. Recently, lack of association was demonstrated between the +49 A/G transition and Crohn's disease in the Czech population, similar to the results of a previous Hungarian IBD study. However, the literature concerning the role of CTLA4 +49 A/G in CD development is disproportionately short, the association of this SNP was dissected extensively in a number of autoimmune diseases; in the majority of the observed autoimmune diseases, the +49 G allele was identified as the predisposing variant, while several studies presented the association of the +49 A allele with celiac disease. There are studies in which the role of the +49 A/G variant could not be reinforced in the autoimmune disease development. In addition the +49 A/G is frequently examined not individually, but in haplotype with other *CTLA4* gene

variants. The *CTLA4* gene is often embedded in haplotype analysis with the neighbouring CD28 and ICOS genes too. Then again it is not rare that the association between the +49 A/G variant and the observed disease pathogenesis was demonstrated only in specific subgroups of patients possessing other well-defined genetic markers, especially specific HLA subtypes. The question arose, that how other genetic factors - disregarded in single gene analyses - alter the participation of this gene variant in forming predisposition to disease development.

IBD5

In the last decade significant association was described between Crohn's disease and a locus of approximately 250kb at 5q31 including a cluster ofcytokine genes like *IL4*, *IL5* and *IL13*. Rioux et al identified 11 haplotype tagging SNPs in this region, comprising the IBD5 risk haplotype. From these markers which are equally associated with CD the IGR2096a_1, IGR2198a_1 and IGR2230a_1 polymorphisms are the most frequently envolved in CD association studies.

AIMS

Our aim was to join the major susceptibility genes, namely the *CARD15*, *ATG16L1* and *IL23R* genes and IBD5 locus into a gene-gene interaction analysis in the Hungarian CD population. The following polymorpisms were analysed for statistical interaction:

1) *CARD15* gene: rs2066844 (R702W), rs2066845 (G908R), rs2066847 (L1007fs)
2) *ATG16L1* gene: rs2241880 (T300A)
3) *IL23R* gene: rs1004819 and rs2201841
4) IBD5 locus: rs1762208 (IGR2230a_1), rs11739135 (IGR2198a_1), rs12521868 (IGR2096a_1)

Additionally we performed a combined genetic analysis, stratifying the *CTLA4* +49 A/G variant by three IBD5 markers (IGR2230a_1, IGR2198a_1 and IGR2096a_1) to test for the possible statistical interactions with respect to possible changes in Crohn's disease risk in the Hungarian population.

MATERIALS AND METHODS

Patients and controls

The statistical interaction of the main CD susceptibility genes (*CARD15*, *ATG16L1*, *IL23R* and IBD5 loci) was determined in 315 unrelated patients with Crohn's disease (151 males, 164 females, mean age 38.65 ± 0.79 years). For the interaction analyses of *CTLA4* gene and IBD5 locus 305 patients were involved (146 males, 159 females, mean age 38.7 ± 0.80 years) in the study. The CD group included mixed Caucasian patients who had typical symptoms and diagnosis. The origin of DNA samples was the central Biobank governed by the University of Pécs, as part of the National Biobank Network of Hungary, which belongs also to the pan-European Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) preparatory phase project. The governance, maintenance and management principles of the Biobank had been approved by the national Scientific Research Ethics Committee, Budapest (ETT TUKEB). Blood samples of patients were collected in Szombathely, Zalaegerszeg, Pécs, Békéscsaba, Miskolc and Budapest. A group of 314 (major CD genes) and 310 (*CTLA4* and IBD5) clinically healthy subjects (170 males, 144 females, mean age 40.8±0.80 years; 169 males, 141 females, mean age 40.8 ± 0.80 years) with no IBD or other autoimmune disease were collected for the study. During the entire investigation period the guidelines and regulations of the 1975 Helsinki Declaration and the currently

operative national laws were followed; the patients gave their informed consent for use of their collected, anonymized DNA samples for research purposes.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes with a routine salting out method; for genotyping PCR-RFLP methods and direct sequencing were used. The PCR products were digested by allele-specific restriction endonucleases Hin1II (rs11739135), TrulI (rs12521868), HinP1I (rs2066845), BspLI (rs2066847), LweI (rs2241880), TaaI (rs1004819) and HpyF3I (rs2201841) DdeI (rs17622208), and BseXI (rs231775). The amplicon contained an obligate cleavage site of the restriction enzyme for the suitable visual control of the efficacy of the digestion. The restriction fragments were separated by electrophoresis on 3% agarose gels containing ethidium bromide and visualized by UV transillumination. The genotyping of G908R (rs2066844) variant in *CARD15* was carried out by direct sequencing.

Statistical analysis

Statistical analysis was carried out using SPSS 15.0. package for Windows. The allele frequencies were compared with Pearson's χ^2 test. Haploview 4.1 was used to test linkage disequilibrium. Binary logistic regression analysis was applied to observe the individual contributions of *CARD15*, *ATG16L1*, *IL23R,* IBD5 and *CTLA4* variants. An association was considered significant if a *P* value of <0.05 was attained. *CARD15* status was classified as – (wild type) or + (at least one mutations in any of the three *CARD15* SNPs). The genetic models used in two-locus analyses were chosen according to the recessive or dominant feature of each observed polymorphism. The following genotypes were regarded as reference: *IL23R* rs1004189 wild type (GG); *IL23R* rs2201841 wild type+heterozygote (TT+TC); *ATG16L1* rs2241880 wild type+heterozygote (AA+AG); *CARD15* R702W wild type (CC); *CARD15* G908R wild type (GG); *CARD15* 1007fs wild type (– –); *CARD15*– status; IGR2230a_1 wild type (GG); IGR2198a_1 wild type (GG); IGR2096a_1 wild type (GG), *CTLA4* wild type+ heterozygote (GA+AA) genotype. The odds ratios and confidence intervals for the genotype combinations were derived from binary logistic regression analyses (using interaction term) and χ^2 test (in 2x2 contingency tables). Since large number of hypotheses were tested during the interaction analyses, the less conservative Benjamini-Hochberg method was applied for multiple hypotheses correction (FDR=0.05).[1]

[1] Data of the Results section contain the original published significancy values (*P)* without Benjamini-Hochberg correction for multiple hypotheses. The subsequent correction of *P* values generally caused only a slight change in the significancy of the results, the lost of significancy is indicated in the text of the thesis.

RESULTS

I. The *IL23R* rs1004819 variant was observed firstly in the Hungarian CD population in this study; the A allele and the AA genotype of this mutation show significant association with Crohn's disease.

II. The results of previous national studies were confirmed: two variants in the *CARD15* gene, namely the R702W and L1007fs, and the T300A mutation of the *ATG16L1* gene confer risk for Crohn's disease in the Hungarian population. The number of subjects enrolled in this study is outsanding concerning the size of Hungary; moreover as the samples were collected from several regions, the whole population of the country was represented.

III. Our previous results were confirmed using enlarged case-control groups as well: rs2201841 variant of *IL23R* gene, and the IBD5 risk haplotype member IGR2198a_1 and IGR2096a_1 markers are risk factors of CD pathogenesis; the *CARD15* G908R mutation, the IBD5 IGR2230a_1 polymorphism, and the *CTLA4* +49A/G variant have neutral effect in disease.

IV. The observed disease risk variants show association with CD independently of each other. Concerning the increasing value of risk caused by the examined SNPs, the variants can be listed in the next ascending order: IGR2096a_1, IGR2198a_1, *ATG16L1* T300A, *IL23R* rs1004819, *CARD15* R702W, *CARD15* status, *CARD15* L1007fs and *IL23R* rs2201841.

V. The analyses of genotype combinations revealed that:

1. The *ATG16L1* T300A mutation in homozygote form confers risk independently of *CARD15* R702W and L1007fs mutations. These two *CARD15* mutations multipled the risk in the presence of the observed *ATG16L1* gene variant; the same effect was detected in the case of the pooled form of the three analysed *CARD15* variant (*CARD15* status).

2. The *ATG16L1* T300A mutation in homozygote form confers risk independently of IGR2198a_1 and IGR2096a_1 markers in the IBD5 locus. Both IBD5 variants increase the disease susceptibility independently of *ATG16L1*. The T300A variant is stronger risk factor when combinated with one of the two IBD5 SNPs than in itself.

3. The two IL23R polymorphisms behave differently if combinated with the *ATG16L1* mutation: the *IL23R* rs2201841 CC and the *ATG16L1* AA homozygote genotype increase disease risk independently, their concurrent presence results in even higher risk. The *IL23R* rs1004189 mutation does not act as a risk factor in default of the *ATG16L1* AA genotype, and *vice versa* the *ATG16L1* AA genotype is not associated with the disease on normal rs1004189 background. The *IL23R* rs1004189 and *ATG16L1* T300A variants together result in higher disease risk compared to their individual effect.

4. The two *IL23R* variants and the *CARD15* R702W and L1007fs mutations are independent CD risk factors, however the combination of *IL23R* rs2201841 CC genotype and *CARD15* R702W risk allele do not show association with the disease.

5. While the *IL23R* rs2201841 mutation increases the risk independently of IBD5 background, the rs1004819 confer risk only when paired with one of the IBD5 markers. Morover the susceptibility effect of IBD5 locus do not work on normal rs1004819 background.

6. The *CARD15* gene and IBD5 cus were found to be independent risk factors in the observed Hungarian CD group. Both IGR2198a_1 and az IGR2096a_1 variants increase CD risk in the presence of normal *CARD15* R702W or L1007fs genotypes. The examined *CARD15* mutations cause strong risk both in the absence and presence of the IBD5 variants.

7. The combination of *+CARD15* status and *IL23R* rs2201841 variant resulted in far the highest relative odds ratio, followed by the slightly lower disease risk of *IL23R* rs2201841 and *CARD15* L1007fs pair.

8. The CTLA4 +49 AA genotype modifies the susceptibility effect of the observed IBD5 markers: the IGR2198a_1 és IGR2096a_1 polymorphisms increase disease risk only in the presence of this CTLA4 genotype. The third IBD5 variant, namely the IGR2230a_1, which possessed neutral feature in single-loci analysis, do not show association with CD either in any combination with the CTLA4 variant.

Table 1 Genotype and allele frequencies of variants in *IL23R*, *ATG16L1*, *CARD15* and IBD5

	CD	Control	OR (95% CI)*	*P*
IL23R (rs1004189)				
GG	119 (37.8%)	151 (48.1%)		
GA	152 (48.3%)	140 (44.6%)		
GA+AA	196 (62.2%)	163 (51.9%)	1.50 (1.09-2.08)	0.013
AA	44 (14.0%)	23 (7.3%)	2.05 (1.20-3.50)	0.008
RAF	0.381	0.296		0.001
IL23R (rs2201841)				
TT	131 (41.6%)	152 (48.4%)		
TC	139 (44.1%)	145 (46.2%)		
TC+CC	184 (58.4%)	162 (52.6%)	1.28 (0.93-1.76)	0.14
CC	45 (14.3%)	17 (5.4%)	2.97 (1.65-5.33)	<0.001
RAF	0.363	0.285		0.003
ATG16L1 T300A (rs2241880)				
AA	56 (17.8%)	72 (22.9%)		
AG	151 (47.9%)	163 (51.9%)		
AG+GG	259 (82.2%)	242 (77.1%)	1.45 (0.98-2.17)	0.06
GG	108 (34.3%)	79 (25.2%)	1.69 (1.19-2.41)	0.004
RAF	0.583	0.511		0.011
CARD15 R702W (rs2066844)				
CC	275 (87.3%)	294 (93.6%)		
CT	38 (12.1%)	18 (5.7%)		
CT+TT	40 (12.7%)	20 (6.4%)	2.13 (1.19-3.80)	0.011
TT	2 (0.6%)	2 (0.6%)	0.62 (0.06-6.98)	0.70
RAF	0.067	0.035		0.011
CARD15 G908R (rs2066845)				
GG	299 (94.9%)	305 (97.1%)		
GC	16 (5.1%)	9 (2.9%)		
GC+CC	16 (5.1%)	9 (2.9%)	1.65 (0.71-3.86)	0.24
CC	0 (0.0%)	0 (0.0%)	n	n
RAF	0.025	0.014		0.17
CARD15 L1007fs (rs2066847)				
– –	264 (83.8%)	291 (92.7%)		
– C	42 (13.3%)	23 (7.3%)		
– C+CC	51 (16.2%)	23 (7.3%)	2.57 (1.51-4.36)	<0.001
CC	9 (2.9%)	0 (0.0%)	n	n
RAF	0.095	0.037		<0.001
CARD15 status				
–	220 (69.8%)	262 (83.4%)		
+	95 (30.2%)	52 (16.6%)	2.17 (1.46-3.21)	<0.001
IGR2198a_1 (rs11739135)				
GG	91 (28.9%)	120 (38.2%)		
GC	160 (50.8%)	144 (45.9%)		
GC+CC	251 (79.7%)	194 (61.8%)	1.54 (1.10-2.15)	0.013
CC	64 (20.3%)	50 (15.9%)	1.32 (0.87-1.99)	0.19
RAF	0.457	0.389		0.014
IGR2096a_1 (rs12521868)				
GG	94 (29.8%)	120 (38.2%)		
GT	149 (47.3%)	142 (45.2%)		
GT+TT	221 (70.2%)	194 (61.8%)	1.44 (1.03-2.02)	0.034
TT	72 (22.9%)	52 (16.6%)	1.45 (0.97-2.17)	0.07
RAF	0.465	0.392		0.009

RAF: risk allele frequency; *adjusted for age and gender.

Table 2 Case-control genotype and allele frequencies of variants in *CTLA4* and IBD5

	CD	Control	OR (95% CI)*	*P*
CTLA4 (rs231775)				
GG	33 (10.8%)	48 (15.5%)		
GA	144 (47.2%)	148 (47.7%)	0.99 (0.72-1.36)	0.954
GA+AA	272 (89.2%)	262 (84.5%)	1.51 (0.94-2.44)	0.088
AA	128 (42.0%)	114 (36.8%)	1.23 (0.89-1.70)	0.213
RAF	0.656	0.606		0.073
IGR2230a_1 (rs17622208)				
GG	71 (23.3%)	91 (29.4%)		
GA	158 (51.8%)	149 (48.1%)	1.13 (0.82-1.56)	0.441
GA+AA	234 (76.7%)	219 (70.6%)	1.35 (0.94-1.95)	0.102
AA	76 (24.9%)	70 (22.6%)	1.16 (0.80-1.69)	0.431
RAF	0.508	0.466		0.140
IGR2198a_1 (rs11739135)				
GG	88 (28.9%)	120 (38.7%)		
GC	155 (50.8%)	140 (45.2%)	1.24 (0.91-1.71)	0.179
GC+CC	217 (71.1%)	190 (61.3%)	1.55 (1.10-2.17)	0.012
CC	62 (20.3%)	50 (16.1%)	1.33 (0.88-2.01)	0.179
RAF	0.457	0.387		0.013
IGR2096a_1 (rs12521868)				
GG	91 (29.8%)	120 (38.7%)		
GT	144 (47.2%)	138 (44.5%)	1.09 (0.79-1.50)	0.592
GT+TT	214 (70.2%)	190 (61.3%)	1.45 (1.03-2.03)	0.032
TT	70 (23.0%)	52 (16.8%)	1.47 (0.98-2.19)	0.062
RAF	0.466	0.390		0.008

RAF: risk allele frequency; *adjusted for age and gender.

Table 3 Pairwise analysis of *IL23R, ATG16L1, CARD15* and *IBD5*: relative OR values

	IL23R rs1004189		*IL23R* rs2201841		*ATG16L1*	
	GG	GA+AA	TT+TC	CC	AA+AG	GG
CARD15 R702W						
CC	1	**1.56 (1.12-2.19)**	1	**3.04 (1.67-5.57)**	1	**1.57 (1.09-2.25)**
CT+TT	**2.35 (1.03-5.34)**	**3.18 (1.45-6.97)**	**2.25 (1.26-4.03)**	4.75 (0.53-42.81)	**2.20 (1.14-4.26)**	**3.18 (1.11-9.08)**
CARD15 G908R						
GG	1	**1.51 (1.09-2.09)**	1	**2.69 (1.49-4.86)**	1	**1.51 (1.06-2.14)**
GC+CC	1.55 (0.46-5.21)	3.23 (0.99-10.57)	1.49 (0.62-3.59)	n	1.44 (0.56-3.72)	6.91 (0.83-57.92)
CARD15 L1007fs						
– –	1	**1.57 (1.12-2.20)**	1	**2.89 (1.57-5.32)**	1	**1.54 (1.07-2.22)**
–C+CC	**2.85 (1.28-6.35)**	**3.40 (1.69-6.82)**	**2.43 (1.42-4.18)**	**8.52 (1.04-69.74)**	**2.37 (1.29-4.34)**	**4.27 (1.54-11.79)**
CARD15 status						
–	1	**1.50 (1.04-2.16)**	1	**2.70 (1.42-5.15)**	1	**1.54 (1.04-2.27)**
+	**2.10 (1.17-3.76)**	**3.33 (1.96-5.67)**	**2.12 (1.42-3.16)**	**9.15 (2.05-40.74)**	**2.12 (1.35-3.31)**	**3.82 (1.86-7.86)**
IGR2198a_1						
GG	1	1.43 (0.80-2.53)	1	**4.83 (1.52-15.37)**	1	1.71 (0.94-3.09)
GC+CC	1.45 (0.84-2.48)	**2.44 (1.43-4.15)**	**1.58 (1.11-2.24)**	**3.66 (1.81-7.41)**	**1.60 (1.07-2.38)**	**2.38 (1.46-3.87)**
IGR2096a_1						
GG	1	1.55 (0.88-2.73)	1	**4.03 (1.39-11.62)**	1	1.63 (0.91-2.92)
GT+TT	1.49 (0.87-2.56)	**2.41 (1.42-4.09)**	**1.50 (1.06-2.13)**	**3.71 (1.80-7.67)**	**1.50 (1.01-2.24)***	**2.32 (1.42-3.79)**
ATG16L1						
AA+AG	1	1.34 (0.92-1.95)	1	**2.67 (1.31-5.44)**	-	-
GG	1.16 (0.68-1.99)	**2.51 (1.55-4.08)**	**1.48 (1.03-2.14)**	**4.68 (1.72-12.78)**	-	-

***P*<0.05**

*not significant after Benjamini-Hochberg correction

Table 4 Pairwise analysis of IBD5 and *CARD15*: relative OR values

	IGR2198a_1		IGR2096a_1	
	GG	GC+CC	GG	GT+TT
CARD15 R702W				
CC	1	**1.60 (1.13-2.27)**	1	**1.55 (1.09-2.20)**
CT+TT	**3.17 (1.15-8.69)**	**2.819 (1.39-5.72)**	**3.07 (1.20-7.86)**	**2.75 (1.32-5.70)**
CARD15 G908R				
GG	1	**1.52 (1.08-2.13)**	1	**1.47 (1.05-2.06)**
GC+CC	1.79 (0.39-8.22)	2.69 (0.97-7.45)	2.19 (0.51-9.41)	2.41 (0.86-6.77)
CARD15 L1007fs				
– –	1	**1.61 (1.13-2.31)**	1	**1.48 (1.03-2.11)**
–C+CC	**3.04 (1.30-7.14)**	**3.58 (1.80-7.16)**	**2.43 (1.06-5.59)**	**3.74 (1.85-7.54)**
CARD15 status				
–	1	**1.63 (1.11-2.39)**	1	**1.54 (1.05-2.26)**
+	**2.65 (1.36-5.17)**	**3.19 (1.90-5.37)**	**2.46 (1.29-4.69)**	**3.18 (1.87-5.39)**

***P*<0.05**

Table 5 Pairwise analysis of *CTLA4* and IBD5: relative OR values

	IGR2230a_1		IGR2198a_1		IGR2096a_1	
	GG	GA+AA	GG	GC+CC	GG	GT+TT
CTLA4						
GA+GG	1	1.19 (0.76-1.87)	1	1.43 (0.93-2.19)	1	1.20 (0.79-1.84)
AA	0.941 (0.490-1.810)	1.59 (0.99-2.57)	1.06 (0.60-1.88)	**1.86 (1.17-2.94)***	0.85 (0.48-1.51)	**1.74 (1.11-2.75)***

***P*<0.05**

*not significant after Benjamini-Hochberg correction

DISCUSSION

Since the identification of NOD2/CARD15 as the first susceptibility gene for Crohn's disease in 2001, several additional loci have been implicated in CD and confirmed by replication, among others the *IBD5*, *IL23R* and *ATG16L1* loci, up to date the number of disease-associated loci expanded to nearly 140. Recently the idea was raised that exploring gene-gene interactions might lead to a better understanding of disease cause and might help the prediction of disease risk. So far numerous studies assessed the risk for the development of CD by combining information from the known genetic risk variants associated with the disease.

ATG16L1 and *CARD15*

Hampe et al. found a modest but significant association between *ATG16L1* and *CARD15* in their pioneer German study. Tthough the *ATG16L1* variant was found to be a risk factor even in the absence of the three *CARD15* mutations, on the background of *CARD15* high-risk pooled genotype, the risk conferred by carrying the *ATG16L1* allele G seemed to be higher than in the presence of low risk *CARD15* profile. Nevertheless the authors underlined the need for larger studies since the confidence intervals of the respective risk ratios were wide owing to the small number of controls with pooled high-risk *CARD15* genotype. Prescott et al. also represented that *ATG16L1* increased the susceptibility for CD irrespective of *CARD15* status, at the same time they detected increased *ATG16L1* G allele frequency in *CARD15* carriers compared with noncarriers, which may indicate a weak interaction between these candidate CD susceptibility genes. In a study from New-Zealand an additive effect was assumed between *ATG16L1* and *CARD15*. Although no epistatic interaction was detected between the two genes in pediatric patients with Crohn's disease, the concurrent presence of these suscptibility alleles increased the risk of disease pathogenesis. The majority of subsequent Caucasian studies failed to demonstrate statistical interaction between the two loci. No evidence of statistical interaction was found between the *ATG16L1* T300A and *+CARD15* status in the study of Büning et al. involving not only German and Dutch patients, but 147 Hungarian CD subjects from Szeged as well. In our study we observed these two genes in a larger group of cases representing not only one region of the country, but the whole CD population of Hungary. We could confirm the independent features of *ATG16L1* T300A and two of the well-known *CARD15* mutations (R702W and L1007fs), morover our results revealed a 3-4-fold increase in disease risk due to the combination of the variants of *ATG16L1*, *CARD15* and *CARD15* status. The interaction of these two Crohn's disease-

associated susceptibility genes is more and more expected according to functional protein-level studies as well. Travassos et al. presented evidence that targeting of bacteria to autophagosomes is enhanced through stimulation of the intracellular censor NOD2 by MDP, and cells expressing the disease variant *ATG16L*1 T300A fail to perform increased autophagy. They found that NOD2 and ATG16L1 colocalize and physically interact at the plasma membrane, and proposed that this interaction allows NOD2 to recruit the autophagy machinery when it senses the presence of invading bacteria. Furthermore, the NOD2 protein encoded by the L1007insC risk allele retains ATG16L1 in the cytosol and prevents the recruitment of the autophagy machinery to bacteria early after infection. The findings of Cooney et al. also could link *CARD15* and *ATG16L1* in a single functional pathway and revealed defects in this pathway in Crohn's disease dendritic cells. Dendritic cells from individuals with Crohn's disease expressing Crohn's disease-associated *NOD2* or *ATG16L1* risk variants are defective in autophagy induction, bacterial trafficking and antigen presentation that could lead to bacterial persistence via impaired lysosomal destruction and immune mediated clearance. The results of Homer et al. also suggest that CD-associated variants of *NOD2* and *ATG16L1* result in defects in an autophagy-dependent antibacterial pathway specifically in epithelial cells. The selective inhibition of different steps of autophagy leaded to decreased activation of signal pathway; diminished NFκB signaling was detected when ATG16L1 was knocked down with iRNA. In contrast to the results of Cooney and Travassos the PBMCs of the *ATG16L1* T300A homozygotes did not differ functionally from the nonhomozygous patients; the MDP-induced TNFα secretion, NOD2 signaling processes, the size and dinamics of autophagy were independent of the genotype. However the *ATG16L1* T300A variant leaded to the loss of NOD2 functionin human colorectal epitelial cell line. Plantinga et al. revealed that the PBMCs of CD patients with *ATG16L1* T300A variant produce elevated levels of the proinflammatory cytokines IL1β and IL6 after stimulated with ligand of NOD2. The *ATG16L1* T300A risk allele is associated with lower protein expression of ATG16L1 after microbial triggering; presumably due to the impaired autophagy activation the NOD2 mediated autophagosome formation (ATG16L1-NOD2 complex formation) and the subsequent antigen presentation is diminished. The authors proposed the modulatory role of ATG16L1 in the balance of NOD2-mediated autophagy induction vs. cytokine production. In the presence of normal ATG16L1 protein the autophagy dominates, while in case of the mutated form the balance is shifted toward RIP2 signaling and subsequent increased IL1β mRNA level. The elevated level of this proinflammatory cytokine could explain the inflammation in Crohn’s disease patients, however concidering the relatively high prevalence

of the *ATG16L1* T300A homozygous genotype and the general sensitivity of NOD2 molecule to virtually all Gram+ and − bacteria in the gut, other immunological factors are expected to be involved in the development of inflammation in CD.

ATG16L1 and IBD5

The majority of Caucasian studies failed to demonstrate statistical interaction between the *ATG16L1* and IBD5 variants in CD: the *ATG16L1* T300A mutation increases disease risk independently of IGR2096a_1 marker, *SLC22A4* C1672T and *SLC22A5* G-207C variants, and the haplotype formed by C1672T and G-207C. In our study we also confirmed this observation in the Hungarian CD population with *ATG16L1*, IGR2198a_1 and IGR2096a_1, morover the *ATG16L1* T300A variant showed higher disease risk when regarded together with one of the IBD5 markers.

ATG16L1 and *IL23R*

One of the two paralel GWA studies describing the *ATG16L1* as a CD susceptibility gene tested for interaction between the new gene variant and the previously identified 13 *IL23R* polymorphisms, but failed to show evidence for association. Parkes et al. combinated the autophagy gene variant only with the *IL23R* rs11805303 SNP but they got similar negative results. In a study from New-Zealand the independent effect of T300A and the rare protective *IL23R* variant R381Q was confirmed. The research group of Cotterill performed a large-scale meatanalysis with 13000 samples, but could not detect significant association between the same gene variants. In a German study the *ATG16L1* T300A and ten variants of the *IL23R* gene, like the R381Q, rs1004189 and rs2201841were tested with no evidence of statictical interaction. In Hungary Lakatos et al. observed the *ATG16L1* T300A and *IL23R* R381Q variants only in single-locus analysis. Althought one of the two *IL23R* gene variants, namely the rs2201841 was also found to be independent of *ATG16L1* T300A mutation in our study, the other variant – tested firstly in the inland CD population – showed an *ATG16L1* dependent behaviour.

IL23R and *CARD15*

Besides the individual risk of *IL23R* variants several studies examined their epistatic interaction with other IBD genes like *CARD15*. Mostly the well-replicated *IL23R* R381Q protecting variant was implied in gene-gene interaction analyses and reported to act independently of *CARD15*. In one study an additive effect was found between this *IL23R* variant and *CARD15* gene, since the R381Q conferred the highest disease risk on the background of the high-risk *CARD15* genotype. The epistasis of the intronic rs1004189 risk

variant with *CARD15* was examined in a German CD population, but no gene-gene interaction was found. Cummings et al. performed interaction analyses between the *CARD15* gene and eight *IL23R* variants but failed to get positive results. In our study high, significant odds ratios were found for the two *IL23R* variants, both in the presence and absence of *CARD15* mutations, suggesting that these genes act also independently on CD risk in the Hungarian population. The combination of *+CARD15* status and *IL23R* rs2201841 variant resulted in far the highest relative odds ratio, followed by the slightly lower disease risk of *IL23R* rs2201841 and *CARD15* L1007fs pair.

_IL23R_ and IBD5

The majority of Caucasian studies dissecting the interaction between these two genes ended without evidence of associations. While Okazaki found just a tendency for interaction between the *IL23R* rs10889677 and IGR2230a_1 variants, the group of Cummings reported an association of the tested *IL23R* variants (rs1004819, rs7517847, rs10489629, rs2201841 és rs1343151) with CD only in the presence of susceptibility haplotype in IBD5. However Glas et al. failed to detect interaction between the *SLC22A4/5* mutations and the *IL23R* rs1004819 variant, in our study for combinations of *IL23R* rs1004189 and IBD5 markers, significant association was seen only in individuals carrying together the rs1004189 mutation and one of the two IBD5 SNPs.We detected significant association in patients bearing *IL23R* rs2201841 CC genotype and wild type IBD5 background together, and vice versa, in carriers of IBD5 variants significantly high risk was detected on *IL23R* rs2201841 nonhomozygous background; accordingly they may play independent role in CD susceptibility.

IBD5 and *CARD15*

Using TDT analyses and case-control groups Mirza et al. described a kind of cooperation between the 5q31 region and the *CARD15* gene: the IBD5 risk haplotype was present only in individuals bearing at least one mutant *CARD15* variant. However in one study the *CARD15* gene and the *OCTN1/2* TC haplotype confers to disease risk together, other studies failed to find significant association between the two loci. In this Hungarian CD population the observed *CARD15* variants and the two IBD5 markers behaved as independent risk factors too.

***CTLA4* and IBD5**

In a recent Czech study a protective effect of a specific *CTLA4* haplotype was unmasked in the observed CD group after stratification for the risk variants in the *CARD15* and *IL23R* genes. Kouki and colleagues found that individuals with the CTLA4 +49 GG

genotype have reduced control of T-cell proliferation compared with AA homozygotes, and this may play role in the development of Grave's disease, and presumably in the pathogenesis of other autoimmune diseases. Mäurer et al. found greater proliferative response of T-cells from donors homozygous for G at position +49. On the basis of these data, the A allele would be expected to act as a protective factor, although it is possible that there are genetic differences in the immunity control of the gastrointestinal tract - which plays an important role in promoting immune tolerance - and in the rest of the immune system. This could explain the different roles of the *CTLA4* +49 A and G variants in celiac disease and other autoimmune diseases like type 1 diabetes. This also may explain that it is the +49 A allele, which seems to be associated with Crohn's disease in two-loci analyses in our study: despite the independence seen between *CTLA4* +49 A/G and the analysed variants in IBD5 locus by logistic regression, the *CTLA4* AA genotype showed increased risk for Crohn's disease in specific combinations with IGR2198a_1 and IGR2096a_1 variants. On the other hand it is not impossible that the *CTLA4* +49 A/G variants only mediate the effect of another real causative gene variant being in strong linkage equilibrium with CTLA4 +49 A/G, when combined with IGRs. Large studies with increased power and case numbers are needed to clarify the exact nature of these possible correlations.

Multi-locus analyses, high risk genotype combinations

Besides combining the most associated CD susceptibility variants by pair, multilocus analyses with three or more loci were also performed. The research group of Latiano tested for interaction using triplets of *IL23R*, *CARD15*, *ATG16L1* and IBD5 loci. However Prescott et al. also failed to detect statistical interaction between the *ATG16L1*, *CARD15* and IBD5 loci, homozygosity for the risk allele at all three loci conferred a combined risk of 20.4 for Crohn's disease. The number of risk alleles of *CARD15, IL23R*, *IRGM* and *PTGER4* is found to be elevated in Ashkenazi Jewish CD patients compared to controls. Weersma et al. desribed an association between the number of risk alleles in *ATG16L1*, *IL23R*, *CARD15*, IBD5 and *DLG5* loci and an increase of disease risk and more severe disease course suggesting that combining information from the known common risk polymorphisms may enable clinicians to predict the course of Crohn's disease. The sufficiently large sample size is the most important limitation factor of multi-locus analyses. Unfortunatelly in our study there was no possibility to combine more than two loci in interaction analyses due to the relatively low case number; but considering the international result it might be good reasoned to plan

multi-locus analyses with increased subject numbers to identify and clarify high risk genotype combinations in the Hungarian CD population.

PUBLICATIONS

Publications related to the thesis (IF: 4.477)

1. **Csöngei V**, Járomi L, Sáfrány E, Sipeky C, Magyari L, Polgár N, Bene J, Sarlós P, Lakner L, Baricza E, Szabó M, Rappai G, Melegh B. Interaction between CTLA4 gene and IBD5 locus in Hungarian Crohn's disease patients. Int J Colorectal Dis 26: 1119-1125 (2011). **IF: 2.385**
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