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Genotypic Interaction and Gender Specificity of Common Genetic Variants in the *p53/mdm2* Network in Crohn's Disease

V. Zimmer[a] T. Widmann[b] M. Müller[b] M.F. Ong[c] J.M. Stein[d]
M. Pfreundschuh[b] F. Lammert[a] K. Roemer[b] G. Assmann[b]

[a]Department of Medicine II and [b]Department of Medicine I and José Carreras Research Center for Immunogenetics, Saarland University Hospital, Homburg/Saar, [c]Institute of Medical Biostatistics, Saarland University, Homburg, and [d]Department of Operative Dentistry and Periodontology, University Hospital Aachen, Aachen, Germany

Key Words
Crohn's disease · Genetics · *p53* · *mdm2* · Apoptosis · Epistasis · Single-nucleotide polymorphism

Abstract
Background/Aims: Defective p53-mediated apoptosis and cell cycle control have been implicated in the immunopathogenesis of Crohn's disease (CD). Since common functional variants of *p53* (SNP72 G/C) and its key negative regulator *mdm2* (SNP309 T/G) have been reported to affect cellular apoptotic and cell cycle arrest capacities, we assessed the effects of these variants on CD susceptibility and their relationship to *NOD2/CARD15* as a well-established genetic CD risk factor. ***Methods:*** The variants SNP72 G/C and SNP309 T/G were genotyped in 149 European CD patients and 478 healthy controls. Subgroup analysis was performed in relation to *NOD2/CARD15* status and to demographic/clinical characteristics. ***Results:*** The *p53* SNP72 CC genotype tended to be less frequent in CD. This reached statistical significance only in the male cohort (0 vs. 7.3%; p = 0.037). Genotype and allele frequencies of both single-nucleotide polymorphisms (SNPs) were otherwise not significantly different. In the combined genotypic analysis, the genotype *p53* SNP72 CC was significantly underrepresented in *mdm2* SNP309 TT homozygotes (0 vs. 9.7%; p = 0.034). No association was observed between *NOD2/CARD15* and the respective SNPs. ***Conclusion:*** We report on a gender-specific protective effect of the low-apoptotic SNP72 CC genotype, and a gender-unrestricted genotypic interaction between SNP309 TT and SNP72 CC, which, for the first time, links sequence variation of the *p53/mdm2* network to CD, independent of *NOD2/CARD15*.

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Introduction

Along with ulcerative colitis, Crohn's disease (CD) represents the main phenotype of inflammatory bowel disease as a chronic relapsing inflammatory disorder of the gastrointestinal tract with high prevalence and incidence rates in Western nations [1]. Though the distinct molecular pathogenesis of CD awaits further refinement, compelling evidence exists as to the pivotal pathogenic role of the precarious balance of appropriate immune responses to constant intestinal antigen pressure in genetically susceptible hosts [2, 3]. This complex interaction between the autologous luminal microflora and mucosal immune defenses calls for tight control mechanisms to prevent overwhelming immune activation and maintain oral antigenic tolerance. Indeed, under normal conditions apoptosis plays an essential role in immune homeo-

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Dr. med. Gunter Assmann, Department of Internal Medicine I
José Carreras Research Center for Immunogenetics
University of Saarland Medical School, Kirrberger Strasse
DE–66421 Homburg/Saar (Germany), Tel. +49 6841 162 3000, Fax +49 6841 162 3069
E-Mail gunter.assmann@uniklinikum-saarland.de

Table 1. Demographic and clinical characteristics of patients with Crohn's disease (CD) and healthy controls

Clinical parameters	CD patients (n = 149)	Controls (n = 478)
Age, years (median)	36.6 ± 9.8 (36)	34.5 ± 11.3 (33)
Gender male/female (%)	71/78 (47.7/52.3)	302/176 (63.2/36.8)
Smoking ongoing/past/never (%)	56/42/51 (37.5/28.2/34.3)	
CD-associated arthritis: yes/no (%)	26/123 (17.5/82.5)	
NOD2-positive/-negative/unknown (%)	65/81/3 (43.6/54.4/2.0)	

stasis, e.g. antigen-activated T cells are driven to programmed cell death after antigen clearance. By contrast, mucosal T cells were demonstrated to be highly resistant to apoptosis in CD related to both the extrinsic and intrinsic pathways [4–6]. An important nodal point of homeostatic mucosal lymphocyte control seems to converge on p53, e.g. in that it slows down the cell cycle, thus physiologically inhibiting undue cellular expansion [7]. Conversely, the T-cell compartment of CD patients has been shown to more rapidly passage through the cell cycle, resulting in T-cell hyperactivation and inappropriate replication, in the end perpetuating chronic intestinal inflammation [8].

The p53 tumor suppressor and its most important negative regulator murine double minute-2 (Mdm2) are central to a pathway that eliminates damaged cells through apoptosis [9]. Of interest, common genetic variants pertaining to *p53*-mediated cell cycle control and apoptosis pathways have been identified. The single-nucleotide polymorphism (SNP)309 T/G (rs2279744) in the promoter/enhancer site of *mdm2* gene in exon 2, the key negative regulator of p53, critically determines the binding efficiency of the transcription factor SP1. The G allele has been reported to produce higher intracellular levels of Mdm2 and thus less functional p53 in stressed cells [10]. The *p53* SNP72 G/C (Arg72Pro; rs1042522), located in a proline-rich domain of the *p53* gene, probably affects the molecular structure of p53 and is considered to influence its functional activity such that the C allele has been reported to induce apoptosis less efficiently and increases cell cycle arrest potential [11].

From this perspective, we hypothesized that genetically determined alterations in the p53/Mdm2 pathways affecting inflammation, apoptosis and cell cycle control may be implicated in CD, and investigated a possible association of two common variants in the *p53/mdm2* network with CD susceptibility and *NOD2/CARD15* status as a well-established genetic CD risk factor.

Methods

Study Participants

We recruited 149 CD patients from inflammatory bowel disease departments on an outpatient basis. Healthy blood donors from the Institute for Transfusion Medicine, University of Saarland Medical School, served as controls (n = 478). Patients and controls were of central European Caucasian ethnicity. The clinical characteristics of the study participants, including age, gender, smoking habits, CD-associated arthritis, and *NOD2/CARD15* positivity, designated as, at least, heterozygosity in either CD-associated SNP (Arg-702Trp, Gyl908Arg, Leu1007finsC) are outlined in table 1. Smoking behavior and evidence for CD-associated arthritis was evaluated by patient interviews and reviews of the medical records.

The study was approved by the local ethics committee, and all individuals in the study gave written informed consent. The study was carried out in accordance with the World Medical Association Helsinki Declaration and its last amendment in 1998. Case patients were diagnosed as unequivocally having CD through standard clinical, endoscopic, radiological and histological findings on the basis of current guidelines [12].

Preparing Genomic DNA and Genotyping

For genotype analysis, peripheral blood from all study participants was collected. Genomic DNA was extracted from peripheral venous blood leukocytes by standard procedures (Qia Amp DNA Blood Mini Kit, Qiagen, Hilden, Germany). DNA was diluted in water to a final concentration of 15 ng/μl, and 5 μl (75 ng) was used per reaction. *p53* and *mdm2* SNP analyses were performed as previously reported [13]. The three common CD-associated *NOD2/CARD15* variants (*Arg*702*Trp*, *Gyl*908*Arg*, *fsins*C1007) were genotyped employing solution-phase hybridization reactions with 5′-nuclease and subsequent fluorescence detection (TaqMan assays, Applied Biosystems, Darmstadt, Germany; primer and probe sequences can be obtained on request). Genotyping was performed twice blinded to clinical data.

Statistical Analysis

Data were analyzed using SPSS and SAS statistical software. The differences in genotype and allele frequencies between patients and controls were analyzed using χ^2 tests for 2 × 3 tables and 2 × 2 tables, respectively, with Fisher's correction, if appropriate (cases of small numbers, tables containing the value zero). Differences in genotype and allele frequencies including data splitting for gender-stratified subgroup analysis were quantified by odds ratios (ORs) and 95% confidence interval (CI). Significance was assumed for p values of <0.05.

Table 2. Genotype frequencies of the *p53* SNP72 and *mdm2* SNP309 in patients with Crohn's disease (CD) (n = 149) and healthy controls (n = 478)

Genotypes	Group		p value
	CD patients (n = 149)	controls (n = 478)	
p53 SNP72			0.240[a]
CC	4 (2.7%)	30 (6.3%)	
GC	60 (40.3%)	185 (38.7%)	
GG	85 (57.0%)	263 (55.0%)	
mdm2 SNP309			0.787[a]
GG	18 (12.1%)	68 (14.2%)	
TG	76 (51.0%)	234 (49.0%)	
TT	55 (36.9%)	176 (36.8%)	

[a] χ^2 tests for 2×3 tables.

Table 3. Allele frequencies of the *p53* SNP72 and *mdm2* SNP309 in patients with Crohn's disease (CD) (n = 149) and healthy controls (n = 478)

	Group		p value (odds ratio) [95% CI]
	CD (n = 298)	control (n = 956)	
SNP72 allele			0.328[a]
C	68 (22.8%)	245 (25.6%)	(0.858)
G	230 (77.2%)	711 (74.4%)	[0.631; 1.166]
SNP309 allele			0.729[a]
G	112 (37.6%)	370 (38.7%)	(0.954)
T	186 (62.4%)	586 (61.3%)	[0.729; 1.247]

[a] χ^2 tests for 2×2 tables.

Results

The genotype distributions for both SNPs were in Hardy-Weinberg equilibrium. Allele and genotype frequencies of CD patients and healthy controls are shown in tables 2 and 3. The analysis of allele and genotype frequencies of *p53* SNP72 G/C and *mdm2* SNP309 T/G revealed no significant difference between CD patients and controls. However, genotype *p53* SNP72 CC tended to be less frequent in CD patients compared to healthy controls (n = 4 [2.7%] vs. 30 [6.3%], p = 0.240). In a subgroup analysis of gender, the difference reached statistical significance in males (0/71 [0%] vs. 22/302 [22%], p = 0.037; table 4). The subgroup analyses of CD patients with regard to smoking habits, CD-associated arthritis, and *NOD2/CARD15* status failed to reveal differences in genotype or allele frequencies of *p53* SNP72 G/C or *mdm2* SNP309 T/G. There were insufficient clinical and follow-up data available in this cohort to establish unequivocal phenotypic classification of CD. Therefore, analysis of genotype-phenotype associations was not possible.

The combined analysis of the gene polymorphisms in CD and controls documented a significantly lower frequency of the homozygous minor *p53* allele genotype in the subgroup of patients carrying the genotype *mdm2* SNP309 TT (0/55 [0%] vs. 17/176 [9.7%] of *p53* SNP72 CC in *mdm2* SNP309 TT groups, p = 0.034) (table 5). Similarly, the *p53* SNP72 C allele within the *mdm2* SNP309 TT group tends to be less frequent (without reaching statistical significance) in CD patients compared to controls (for *mdm2* SNP309 TT groups 23/87 [20.9%] vs. 97/255 [27.6%] C alleles [*p53*], p = 0.165; table 6).

The reciprocal analysis of *mdm2* SNP309 TT in *p53* SNP72 CC groups of CD patients and controls failed to show differences at a statistically significant level (0/4 [0%] vs. 17/30 [56.7%], p = 0.070; table 5). Furthermore, differences in the frequency of *mdm2* SNP309 T allele in the *p53* SNP72 CC group did not reach significance (for *p53* SNP72 CC groups n = 3/8 [37.5%] vs. 44/60 [73.3%] T alleles *(mdm2)*, p = 0.096; table 6).

Discussion

NOD2/CARD15, engaged in intracellular bacterial sensing and processing, is the first identified CD-susceptibility gene [14, 15]. Since its discovery, unprecedented progress in delineating the genetic architecture of CD as a complex trait with high heritability has been achieved. With the recent implementation of large-scale genome-wide association studies, new genetic susceptibility factors have been identified, and, owing to subsequent fine mapping, novel pathways in CD pathogenesis have emerged, e.g. defective autophagy [16] and IL-23R signaling [17]. At the same time, it is apparent that the currently proposed and/or confirmed susceptibility genes amount to only a fraction of disease heritability, such that additional genetic factors await their identification [18].

Despite its relevant pathogenic role in CD, a putative genetic basis of aberrant apoptosis and cell cycle control

Table 4. Gender distrubution of genotype frequencies of the *p53* SNP72 and *mdm2* SNP309 in patients with Crohn's disease (CD) (n = 149) and healthy controls (n = 478)

Gender	Genotypes	Group		p value
		CD patients (n = 149)	controls (n = 478)	
Female	*SNP72*			0.977[a]
	CC	4 (5.1%)	8 (4.5%)	
	GC	29 (37.2%)	65 (36.9%)	
	GG	45 (57.7%)	103 (58.5%)	
	SNP309			0.803[a]
	GG	10 (12.8%)	28 (15.9%)	
	TG	40 (51.3%)	89 (50.6%)	
	TT	28 (35.9%)	59 (33.5%)	
	All female	78 (100.0%)	176 (100.0%)	
Male	*SNP72*			0.037[b]
	CC	0 (0.0%)	22 (7.3%)	
	GC	31 (43.7%)	120 (39.7%)	
	GG	40 (56.3%)	160 (53.0%)	
	SNP309			0.875[a]
	GG	8 (11.3%)	40 (13.2%)	
	TG	36 (50.7%)	145 (48.0%)	
	TT	27 (38.0%)	117 (38.7%)	
	All male	71 (100.0%)	302 (100.0%)	

[a] χ^2 tests for 2×3 tables; [b] Fisher exact tests for 2×3 tables.

Table 5. Combined analysis of genotype frequencies in (**A**) the SNP72 G/C and (**B**) SNP309 T/G comparing patients with Crohn's disease (CD) (n = 149) with healthy controls (n = 478)

	Group		p-value
	CD (n = 149)	Controls (n = 478)	
A SNP309 genotypes			
GG			0.908[a]
SNP72 genotypes			
CC	1 (5.6%)	3 (4.4%)	
GC	8 (44.4%)	26 (38.2%)	
GG	9 (50.0%)	39 (57.4%)	
all SNP309 GG	18 (100%)	68 (100%)	
TG			0.888[a]
SNP72 genotypes			
CC	3 (3.9%)	10 (4.3%)	
GC	29 (38.2%)	96 (41.0%)	
GG	44 (57.9%)	128 (54.7%)	
all SNP309 TG	76 (100%)	234 (100%)	
TT			0.034[b]
SNP72 genotypes			
CC	0 (0.0%)	17 (9.7%)	
GC	23 (41.8%)	63 (35.8%)	
GG	32 (58.2%)	96 (54.5%)	
all SNP309 TT	55 (100%)	176 (100%)	
B SNP72 genotypes			
CC			0.070[b]
SNP309 genotypes			
GG	1 (25.0%)	3 (10.0%)	
TG	3 (75.0%)	10 (33.3%)	
TT	0 (0.0%)	17 (56.7%)	
all SNP72 CC	4 (100.0%)	30 (100.0)	
CG			0.833[a]
SNP309 genotypes			
GG	8 (13.3%)	26 (14.1%)	
TG	29 (48.3%)	96 (51.9%)	
TT	23 (38.3%)	63 (34.1%)	
all SNP72 CG	60 (100%)	185 (100%)	
GG			0.611[a]
SNP309 genotypes			
GG	9 (10.6%)	39 (14.8%)	
TG	44 (51.8%)	128 (48.7%)	
TT	32 (37.6%)	96 (36.5%)	
all SNP72 GG	85 (100%)	263 (100%)	

[a] χ^2 tests for 2×3 tables; [b]Fisher exact tests for 2×3 tables.

at the level of p53 has not yet been specifically addressed. In line with such functional candidacy, this is the first association study examining sequence variation in the *p53/mdm2* network in CD, represented by the functional polymorphic *p53* SNP72 G/C and *mdm2* SNP309 T/G. We report on a significant genotype-specific sensitizing effect of p53-Arg72 (expressed from SNP72 G) and/or protective effect of p53-Pro72 (expressed from SNP72 C) in males of central European Caucasian ethnicity. In accord with a sensitizing effect of p53-Arg72, this p53 was more frequently associated with the *mdm2* SNP309 TT genotype that produces less of the p53-antagonist Mdm2, suggesting an epistatic interaction between these two functionally coupled genes. It might be speculated that both the observed gender specificity and the genotypic interaction might be accounted for by alterations in intracellular Mdm2 levels counteracting p53. Since the G allele of SNP309 and, potentially, estrogen signaling may increase cellular Mdm2 availability, the attenuation of p53-mediated apoptosis related to SNP72 CC might, therefore, become abrogated in such genetic and/or gender context [19]. By contrast, neither the *mdm2* gene SNP309 T/G genotype nor the allele frequencies of either SNPs were associated with CD. In addition, stratification for *NOD2/CARD15* positivity yielded no significant cor-

Table 6. Combined analysis of allelic frequencies of the *p53* SNP72 G/C in the *mdm2* SNP309 TT group (**A**) and SNP309 T/G in the SNP72 CC group (**B**) comparing patients with Crohn's disease (CD) (n = 149) with healthy controls (n = 478)

	Group		p value	Odds ratio (95% CI)
	CD patients	controls		
A SNP309 TT genotype	(n = 110)	(n = 352)		
SNP72 allele				0.695 (0.415; 1.164)
C	23 (20.9%)	97 (27.6%)	0.165[b]	
G	87 (79.1%)	255 (72.4%)		
B SNP72 CC genotype	(n = 8)	(n = 60)		
SNP309 allele				4.583 (0.981; 21.411)
G	5 (62.5%)	16 (26.7%)	0.096[b]	
T	3 (37.5%)	44 (73.3%)		

[b] Fisher exact tests for 2×2 tables.

relation with either SNP. However, the overall limited size of our cohort has to be addressed critically, although this has to be balanced against the highly polymorphic minor allele frequencies of the addressed variants. The G allele frequencies of SNP72 and SNP309 have been reported to be 23 (*dbSNP* database) and 35% [20] in a European-based population, ratios comparable to our results in a 'hypernormal' control of healthy blood donors (26 and 39%, respectively). Nevertheless, due to limitations in sample size, definitive formal exclusion of a type-2 error with respect to our globally negative association result and a potential type-1 error for the significant associations in the respective subgroups is not possible.

Since pro-apoptotic p53-Arg72 is over-represented, these findings do not support our initial hypothesis that these SNPs may constitute the basis of aberrant p53-mediated T-cell apoptosis in CD. The precise mechanisms whereby these variants and/or its interaction may modulate CD susceptibility have to be worked out. In addition to its traditional key role as the major tumor suppressor, p53 is increasingly being appreciated as also involved in inflammatory stress responses. For instance, p53 has been reported to modulate NF-κB pathways, and p53-related apoptosis may be linked to NF-κB [21–23]. However, at present, the precise mechanisms and functional consequences of such p53/NF-κB cross talk, and its putative impact, if any, of the SNP72 variant on such p53 function, remain unclear.

Collectively, our findings in a comparatively small cohort of Caucasian European-based CD patients for the first time implicate common genetic variations of the *p53/mdm2* network in CD susceptibility. Clearly, studies on larger, clinically well-characterized cohorts are needed to confirm and extend these findings.

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