

CARD15 Mutations and Perianal Fistulating Crohn's Disease: Correlation and Predictive Value of Antibiotic Response

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Abstract

Background *CARD15* mutations alter bowel immunity and increase susceptibility to Crohn's disease (CD). However, the relation between these mutations and Crohn's perianal fistulas has not been fully clarified.

Aims To assess whether *CARD15* mutations are associated with risk of developing Crohn's perianal fistulas and whether these mutations are predictors of the response of perianal fistulas to antibiotics.

Methods *CARD15* mutations were investigated in 203 consecutive CD patients. Presence/absence of history of perianal fistula was recorded. Patients with history of perianal fistula were divided into two groups (with/without *CARD15* mutations), and response to antibiotics was evaluated in both groups.

Results Of the 203 patients, 60 (29.6%) showed at least one *CARD15* mutation and 55 (27.1%) had history of perianal fistula. History of perianal fistula was identified in 13 (21.7%) patients with mutations and in 42 (29.4%)

patients without mutations ($P = 0.260$). Mean age at diagnosis of first perianal fistula was similar in patients with/without *CARD15* mutations (28.7 ± 9.8 versus 29.7 ± 10.1 years, $P = 0.758$). Average time between disease onset and diagnosis of first perianal fistula was also similar in the two groups (4.6 ± 5.1 versus 5.0 ± 5.9 years, $P = 0.816$). Response of perianal fistulas to antibiotics (metronidazole alone or combined with ciprofloxacin) was significantly higher in patients without *CARD15* mutations (7.7% versus 40.5%, $P = 0.041$).

Conclusions In CD, *CARD15* mutations are not associated with risk of developing perianal fistulas or with time of their outbreak. Nevertheless, patients with perianal fistulas and *CARD15* mutations showed worse response to antibiotics.

Keywords *CARD15* · Crohn's disease · Perianal fistulas · Antibiotics

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Introduction

The transmural inflammation characteristic of Crohn's disease (CD) predisposes patients to formation of fistulas. Up to 43% of CD patients will develop perianal fistulas, with a significant increase of disability and direct and indirect costs, and a decrease in perceived quality of life [1–4]. The risk of developing Crohn's perianal fistulas increases when the disease involves the distal bowel. Patients with colonic CD, particularly those with rectal involvement, have significantly higher incidence of perianal fistulas than patients without colonic involvement [2]. Indeed, only 12% of patients with isolated ileal disease develop perianal fistulas, compared with 92% of patients with rectal involvement [2]. These results were recently

corroborated by a large cross-sectional study carried out in our country [5].

The American Gastroenterological Association technical review on perianal CD recommended division of fistulas into either simple or complex [6]. A simple fistula is superficial (intersphincteric or low transsphincteric), painless, with a single external opening and no evidence of rectovaginal involvement or anorectal stricture [6]. In contrast, a complex fistula is located high (high transsphincteric, extrasphincteric or suprasphincteric), may be associated with pain, can potentially involve multiple external openings, and may be associated with rectovaginal fistula and/or anorectal stricture [6]. This is a clinically important differentiation, because several studies have shown better outcomes for patients with simple fistula tracts [7–10].

Although treatments for fistulizing perianal CD have evolved greatly over the past decade, antibiotics remain widely used and are the recommended first-line treatment for these patients, despite the absence of controlled studies to support this approach [4, 11]. However, the response rate of perianal fistulas to antibiotics is only around 50% [12–16]. Furthermore, ineffective or inappropriate treatment of perianal fistula may lead to recurrence of the fistula or can result in the fistula progressing from simple to complex form [17]. Complex fistulas are more difficult to treat, have decreased healing rates, and are associated with less successful outcomes [7–10]. For these reasons, it would be very important to have predictors of the response of perianal fistulas to antibiotics.

The specific pathogenesis of Crohn's perianal fistulas is unknown, but luminal bacteria seem to play a role in their development and maintenance [18]. Since *CARD15* mutations increase susceptibility to CD and appear to change the host response to luminal bacteria, it is reasonable to speculate on a possible connection between this gene and perianal fistulas [19–24]. However, there are few studies on the correlation between *CARD15* mutations and development of Crohn's perianal fistulas, and they provided conflicting data [25, 26]. There is also limited information on whether *CARD15* variants may influence the response of perianal fistulas to treatment, namely to antibiotics [16]. In this study, we assessed whether *CARD15* mutations are associated with risk of developing Crohn's perianal fistulas and whether these mutations are predictors of the response of perianal fistulas to antibiotics (metronidazole and/or ciprofloxacin).

Methods

Patients

Two hundred three consecutive patients with CD [87 males, age (mean \pm standard deviation) = 35.9 \pm 10.2 years;

116 females, age (mean \pm standard deviation) = 37.1 \pm 7.6 years] were included in this investigation. Diagnosis of CD was based on accepted clinical, endoscopic, radiologic, and histologic criteria [27].

Ethical approval for this research was given by the local Ethics Committee. Each patient was informed of the nature of the study and signed the informed consent form, in accordance with the Declaration of Helsinki.

Sample DNA Extraction

Blood samples were collected with standard venipuncture technique using ethylenediamine tetraacetic acid (EDTA)-containing tubes. Genomic DNA was isolated from whole blood using the QIAamp Mini Kit (QIAGEN GmbH, Germany).

Genotyping

All participants were genotyped for the three major *CARD15* mutations: R702W (SNP8), G908R (SNP12), and 3020insC (SNP13). Genotyping was performed by real-time polymerase chain reaction (PCR): variants R702W and G908R using HybProbe (FRET), and variant 3020insC using SimpleProbe. R702W variant was amplified and detected using the forward primer 5'-AgCCgCACAACTTAgATCAC-3', the reverse primer 5'-gCgggCACAggCATAgC-3', the anchor probe 5'-LC Red640-gTCTggCACTC AgCCAgCaggCCCC-PH, and the donor probe 5'-gCgC CAgAgCaggCCTTCTCA-FL. For assay of the G908R mutation, 5'-gCACATATCaggTACTACTgACACT-3' was used as the forward primer, 5'-TTACCTgAgCCACCT CAAgC-3' as the reverse primer, 5'-LC Red705-CTgAA AAggCCAAAGAgTCAACAgAC-PH as the anchor probe, and 5'-CCACTCTgTTgCCCCAgAA-FL as the donor probe. To detect the 3020insC variant, PCR was carried out using the forward primer 5'-gACAggTgggCTTCAgTAgA-3', the reverse primer 5'-TgAggTTCggAgAgCTAAAACAg-3', and the simple probe 5'-CTgCaggCCCCTTgAAAg-FLQ.

The reaction mixture consisted of 18 μ l PCR master mix plus 2 μ l DNA of each patient. The PCR master mix for the R702W variant included 5.0 mM MgCl₂, 1 \times LightCycler DNA master hybridization probes (Roche, Mannheim, Germany), 0.5 μ M each primer, and 0.1 μ M each of fluorescein and LC-Red-640-labeled probe. Cycling parameters consisted of 1 cycle of 95°C for 2 min, followed by amplification for 45 cycles of 95°C for 0 s, 55°C for 10 s, and 72°C for 5 s. A melting curve was created by measuring the fluorescence signal generated with the following profile: 95°C for 5 s, 50°C for 10 s, and 90°C for 0 s with a slope 0.4°C/s transition. The PCR master mix for the G908R mutation included 2.5 mM MgCl₂, 1 \times LightCycler

DNA master hybridization probes (Roche, Mannheim, Germany), 0.5 μM each primer, and 0.2 μM each of fluorescein and LC-Red-705-labeled probe. Cycling parameters consisted of 1 cycle of 95°C for 2 min, followed by amplification for 40 cycles of 95°C for 10 s, 57°C for 8 s, and 72°C for 17 s. A melting curve was created by measuring the fluorescence signal generated with the following profile: 95°C for 20 s, 40°C for 20 s, and 85°C for 0 s with a slope 0.2°C/s transition. Finally, the PCR master mix for the 3020insC variant included 2.5 mM MgCl₂, 1 \times LightCycler DNA master hybridization probes (Roche, Mannheim, Germany), 0.2 μM forward primer, 0.5 μM reverse primer, and 0.2 μM simple probe. Cycling parameters consisted of 1 cycle of 95°C for 2 min, followed by amplification for 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 20 s. A melting curve was created by measuring the fluorescence signal generated with the following profile: 95°C for 20 s, 40°C for 20 s, and 85°C for 0 s with a slope 0.2°C/s transition. Sterile water was used as a negative control. The change of fluorescence was converted to a melting peak (T_m) by plotting the negative derivative of the fluorescence signal corresponding to the temperature ($-dF/dT$) using the LightCycler software.

The sequence variations of the PCR products were confirmed by DNA sequencing.

Medical History And Outcomes

This information was obtained from the inflammatory bowel disease database of our department and complemented by a review of patients' files. Initially we recorded whether patients developed perianal fistulas before or during follow-up. Then, in patients with history of perianal fistula, we recorded the following: gender, current age, disease location, type of fistulas (simple or complex), age at diagnosis of first perianal fistula, time between disease onset and diagnosis of first perianal fistula, antibiotics used in treatment of perianal fistulas, concomitant medication, and clinical response of perianal fistulas to antibiotic therapy.

Antibiotic therapy was defined as use of metronidazole and/or ciprofloxacin for at least 1 week. Metronidazole was used in all patients (dosage: median 980 mg/day, range 750–1,500 mg/day) and 28 patients also received ciprofloxacin (dosage: median 970 mg/day, range 500–1,000 mg/day).

Response was defined as absence of fistula drainage, even after compression, for at least 4 weeks, according to the Fistula Drainage Assessment definition of remission and following the current recommendations for efficacy endpoints in clinical trials [28, 29].

Clinical and laboratory investigators were blinded to genetic results and to clinical data, respectively.

Definition of Groups

Patients with history of perianal fistula were divided into two groups according to presence or absence of *CARD15* variants (with/without *CARD15* mutations). These groups were compared for: age at diagnosis of first perianal fistula, time between disease onset and diagnosis of first perianal fistula, and clinical response of perianal fistulas to antibiotic therapy.

Statistical Analysis

Categorical variables were expressed as frequency and percentage, and corresponding contingency tables were analyzed with Pearson's chi-square test or Fisher's exact test, as appropriate. Odds ratios (OR) were determined with 95% confidence intervals (95% CI).

Continuous variables were summarized using mean \pm standard deviation. These variables were tested for normal distributions using the Kolmogorov-Smirnov test. The Student's *t* test was employed to compare means of continuous variables and normally distributed data; otherwise, the Mann-Whitney *U* test was applied.

Only *P* values <0.05 were considered significant.

All variants studied were in Hardy-Weinberg equilibrium.

Data were analyzed using the Statistical Package for Social Sciences (SPSS version 17.0; SPSS Inc., Chicago, IL, USA) software for Windows.

Results

A total of 203 patients were studied. *CARD15* variants were detected in 60 patients (29.6%), comprising 28 heterozygotes for SNP8 (13.8%), 13 heterozygotes for SNP13 (6.4%), 12 heterozygotes for SNP12 (5.9%), 3 compound heterozygotes [2 (1.0%) for SNP8/SNP12, 1 (0.5%) for SNP12/SNP13], and 4 homozygotes [2 (1.0%) for SNP13, 1 (0.5%) for SNP8, 1 (0.5%) for SNP12]. History of perianal fistula was identified in 55 (27.1%) patients, including 13 (21.7%) of the 60 patients with mutations and 42 (29.4%) of the 143 patients without mutations ($P = 0.865$).

Mean age at diagnosis of first perianal fistula was similar in the two groups (28.7 ± 9.8 years in patients with *CARD15* mutations versus 29.7 ± 10.1 years in patients without *CARD15* mutations, $P = 0.758$). Average time between disease onset and diagnosis of first perianal fistula was also similar in the two groups (4.6 ± 5.1 years in patients with *CARD15* mutations versus 5.0 ± 5.9 years in patients without *CARD15* mutations, $P = 0.816$).

The overall clinical response of perianal fistulas to antibiotics was 32.7% (18 of the 55 patients). This response was observed in 1 patient with *CARD15* mutation and in 17

patients without *CARD15* mutations (7.7% versus 40.5%, $P = 0.041$) (Table 1). The odds ratio, calculated for the probability of response to antibiotics in patients without mutations compared with patients with mutations, was 8.16 (95% confidence interval 0.97–68.74). This means that the probability of response of perianal fistulas to antibiotics in CD patients without *CARD15* mutations is 8.16 higher than that of patients with mutation of this gene (Table 1). The only patient with *CARD15* mutation who responded to antibiotic therapy was a heterozygote for SNP8. The antibiotics used, as well as the dosage and duration of therapy, were similar in patients with and without *CARD15* mutations (Table 2). Likewise we did not detect any significant differences in concomitant medication (Table 3). Finally, we found that patients with and without *CARD15* mutations did not significantly differ with regard to proportion of simple/complex fistulas (1.5 in patients with *CARD15* mutations versus 1.7 in patients without *CARD15* mutations, $P = 0.921$) or incidence of rectal mucosa involvement by CD (66.6% and 55.0% in patients with and without *CARD15* mutations, respectively; $P = 0.519$).

The 37 patients with perianal fistulas who did not respond to antibiotics then received the following treatments: surgery (26 patients), infliximab (8 patients), and combination surgery + infliximab (3 patients).

Discussion

Perianal fistulas are a common manifestation of CD that can lead to significant morbidity and even proctectomy [1–4].

As it is generally assumed that CD is a heterogeneous disease that is composed of subsets of disorders representing different pathogenic mechanisms, numerous genotype–phenotype studies have been performed reporting the association of *CARD15* variants with multiple characteristics of localization and behavior of CD [25, 26, 30–36]. A major problem in comparing these studies is the heterogeneity of phenotypic classifications presented, especially with regard to penetrating behavior. Some classification schemes, according to the Vienna classification, include perianal fistulas in fistulizing behavior, whereas others, following the

Montreal classification, regard anal involvement as a different entity, separate from intra-abdominal penetrating disease [25, 26, 30–38].

Few studies have specifically evaluated the correlation between *CARD15* mutations and Crohn's perianal fistulas, and their results were controversial. Henckaerts et al., in a recent study involving 875 CD patients, concluded that absence of *CARD15* variants was independently associated with development of perianal fistulas [25]. In contrast, Annese et al. genotyped 360 consecutive CD patients for the three main *CARD15* mutations and found, by univariate analysis, that patients who were carriers of any of the *CARD15* variants presented significantly higher incidence of perianal disease [26]. Additionally, on multivariate analysis, they observed that the R720W and G908P variants were positively and negatively associated with perianal disease, respectively [26]. In our study, we did not find any association between *CARD15* mutations and risk of developing Crohn's perianal fistulas, or with time of perianal fistula outbreak.

Fistula pathogenesis remains poorly understood, and therefore management of patients with Crohn's perianal fistulas remains challenging. Nevertheless, there is evidence suggesting that luminal bacteria might play a role in development and maintenance of fistulas [18, 39–41]. Perhaps for this reason, antibiotics are a widely used and recommended first-line treatment for fistulas in CD patients, despite the near absence of randomized controlled trials supporting this approach [4, 11]. As only 50% of patients respond to this therapy, predictors for selection of patients for antibiotic treatment are crucial [12–16]. This is even more relevant if we remember that inadequate treatment of perianal fistula may lead to fistula recurrence or can result in the fistula progressing from simple to complex form [17].

Regarding the response of perianal fistulas to antibiotics, our results show that this is positively associated with absence of *CARD15* mutations (Table 1). It may be thought that the best response to antibiotics observed in these patients could be related to factors other than *CARD15* genotype [2, 42–44]. This was ruled out by demonstrating no significant differences between patients with and without *CARD15* variants in relation to other factors with a

Table 1 *CARD15* mutations versus response of perianal fistulas to antibiotics

	Total, <i>n</i>	Response of perianal fistulas to antibiotics		<i>P</i>	OR (95% CI)*
		Yes, <i>n</i> (%)	No, <i>n</i>		
Patients with <i>CARD15</i> mutations	13	1 (7.7)	12	0.041	8.16 (0.97–68.74)
Patients without <i>CARD15</i> mutations	42	17 (40.5)	25		

OR odds ratio, CI confidence interval

* Calculated for the probability of response to antibiotics in patients without mutations compared with patients with mutations

Table 2 Characteristics of antibiotic therapy: patients with *CARD15* mutations versus patients without *CARD15* mutations

	With <i>CARD15</i> mutations	Without <i>CARD15</i> mutations	<i>P</i>
Ciprofloxacin alone (% of patients)	0	0	–
Metronidazole alone (% of patients)	44.4	50	0.803
Ciprofloxacin and metronidazole (% of patients)	55.6	50	0.783
Ciprofloxacin: dosage (mg/day)	1,000 ± 0	961 ± 138.7	0.551
Metronidazole: dosage (mg/day)	1,138 ± 356	932 ± 371	0.160
Ciprofloxacin: duration (weeks)	6.0 ± 2.4	6.1 ± 3.3	0.963
Metronidazole: duration (weeks)	8.0 ± 7.0	9.2 ± 8.0	0.687

Table 3 Concomitant medication: patients with *CARD15* mutations versus patients without *CARD15* mutations

	With <i>CARD15</i> mutations (%)	Without <i>CARD15</i> mutations (%)	<i>P</i>
Without medication	25.0	39.5	0.431
5-ASA	75.0	58.1	0.375
Steroids	25.0	7.0	0.148
Azathioprine, <3 months	9.0	19.0	0.251
Azathioprine, ≥3 months	25.0	9.0	0.162

5-ASA 5-aminosalicylic acid

potential effect on the response. In fact, we found no significant differences between these two groups of patients with regard to the characteristics of antibiotic therapy (Table 2), concomitant medication (Table 3), proportion of simple/complex fistulas or incidence of rectal mucosa involvement by CD.

Angelberger et al., in a prospective study including 52 CD patients with perianal fistulas treated with antibiotics, also found better clinical response in patients without *CARD15* mutations [16]. Nevertheless, in that work ciprofloxacin was used as first-line treatment, while in our study all patients received metronidazole, with or without ciprofloxacin (association with ciprofloxacin in about half of the cases). Thus, to our knowledge, our study is the first to show that *CARD15* genotype predicts the response of Crohn's perianal fistulas to treatment with metronidazole alone or in combination with ciprofloxacin. Based on our results, we suggest that antibiotics should be questioned as first-line treatment in perianal fistulas of CD patients with *CARD15* variants.

Usually infliximab is recommended as second-line treatment for Crohn's perianal fistulas when first-line treatment (antibiotics and/or azathioprine/6-mercaptopurine) fails [4, 11]. Nevertheless, given the low response rate of Crohn's perianal fistulas to antibiotics and/or azathioprine/6-mercaptopurine and the encouraging results of infliximab in this setting, some authors consider a “top-down” approach as an option for these patients [4, 11–16]. Our results, demonstrating that *CARD15* genotype seems to predict clinical response of perianal fistulas to antibiotics, suggest that *CARD15* genotype may be a factor in selection

of patients with perianal fistulas for a “top-down” approach. This interesting hypothesis needs to be tested in randomized controlled studies.

There is, so far, no explanation for our observation of a greater response to antibiotics in Crohn's perianal fistulas of *CARD15* wild-type patients. However, we know that *CARD15* plays a role in bowel immunity by participating in recognition of intestinal bacteria [21–24]. Thus, we can speculate that, in *CARD15* wild-type patients, endogenous antimicrobial activity may synergize with antibiotics to enhance their action, while in patients with *CARD15* mutations this synergy does not occur. This speculation is reinforced by some recent data that support an association between *CARD15* mutations and increased risk of several infectious complications [45–47].

Some limitations of our study should be mentioned. Firstly, characterization and evaluation of fistulas were carried out retrospectively. Secondly, the size of the study population is relatively small. Finally, this work was performed in an academic referral hospital; therefore, our results may not be applicable to institutions with different patient populations.

Our work shows how gene identification strategies combined with genotype–phenotype correlations might identify prognostic markers to guide disease management. In the future, randomized controlled studies are highly warranted to confirm our results and to identify more prognostic markers in order to define the optimum approach for treatment of this challenging, and often serious, complication of CD.

In conclusion, our study suggests that *CARD15* mutations are not associated with risk of developing Crohn's perianal fistulas, or with time of perianal fistula manifestation. Nevertheless, in our series, *CARD15* genotype seems to predict clinical response of perianal fistulas to antibiotic treatment. In fact, CD patients with perianal fistulas and *CARD15* mutations showed worse response to antibiotic therapy with metronidazole alone or in combination with ciprofloxacin. Thus, in the light of our findings, we propose that antibiotics should be questioned as first-line treatment for Crohn's perianal fistulas in patients with *CARD15* mutations. Such patient selection might avoid delay in application of effective treatment and minimize the risk of adverse events under antibiotic treatment.

Conflicts of interest statement We declare that we have no conflicts of interest.

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