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ORIGINAL ARTICLE

CARD15 mutations and colorectal cancer in a South European country

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Abstract
Purpose *CARD15* mutations are associated with higher susceptibility to Crohn's disease (CD) and longstanding colonic CD increases the risk of developing colorectal cancer (CRC). The relation between these mutations and sporadic CRC remains controversial. The aim of this study was to assess whether germline and/or somatic *CARD15* mutations are risk factors for sporadic CRC in Portugal and whether there are genotype–phenotype correlations in these patients.
Methods The three main *CARD15* mutations (R702W, G908R and 3020insC) were researched in 112 sporadic CRC patients and 152 healthy subjects.
Results Overall, *CARD15* mutations were found in 18 patients (16.1%) and in 15 controls (9.9%; p=0.132). Individually, the incidence of R702W was significantly higher in patients than in controls (12.5% vs. 5.3%, p= 0.035), whereas the genotype frequencies for G908R (2.7% vs. 3.3%) and 3020insC (0.9% vs. 1.3%) were not statistically different between the two groups. Entire genotypic agreement was found in patients genotyped for blood and neoplastic DNA. A significantly higher incidence of *CARD15* mutations was detected in patients with CRC diagnosed under 60 years old (28.6% vs. 10.4%, p= 0.015) and in female patients (24.4% vs. 10.4%, p= 0.048). No associations were found between *CARD15* mutations and family history, symptoms or CRC pathologic characteristics.
Conclusions The *CARD15* R702W variant might be a predisposing factor to sporadic CRC in Portugal, particularly in patients under 60-years old and in female patients. This susceptibility appears to be linked with germline *CARD15* mutations. Nevertheless, we have found no evidence that *CARD15* mutations predict the pathologic characteristics of CRC.

Keywords Colorectal cancer · *CARD15* · Inflammation · Susceptibility

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Introduction

In developed countries, colorectal cancer (CRC) is one of the most frequent malignancies and is the second most common cause of malignant deaths, preceded only by lung cancer [1, 2]. The incidence of CRC in Portugal was 77 new cases per 100,000 inhabitants in 2006, one of the highest in the Western world [3].

Germline mutations in tumour-suppressor adenomatous polyposis coli genes and DNA mismatch repair genes lead to the recognised familial adenomatous polyposis related CRC and hereditary non-polyposis CRC, respectively [4]. These inherited cases account for about 5–10% of CRC [4]. Excluding inherited types of CRC, the susceptibility of a particular individual to developing sporadic CRC remains largely undetermined. Nevertheless, the pathogenesis of sporadic CRC is thought to be multifactorial, involving multiple genetic and various environmental factors [5–7].

Bacterial flora keeps the normal colon mucosa in a continuous state of low-grade inflammation, stimulating the release of various pro-inflammatory cytokines by the immune cells [8]. Several epidemiological and biological data support a clear association between chronic inflammation and cancer [9]. Typical examples are the causal connection between *Helicobacter pylori*-induced chronic gastritis and the development of mucosal-associated lymphoid tissue (MALT) B cell lymphoma and/or gastric cancer [10], as well as the association between longstanding inflammatory bowel disease (IBD) and CRC [11]. In fact, it is estimated that up to 15% of all IBD patients will develop CRC [11]. Furthermore, precursor CRC lesions may often have inflammatory histological features [8, 12] and pro-inflammatory genes have been shown to be important in the maintenance and progression of CRC [13]. In addition, inflammation may favour tumourigenesis by inducing DNA damage [14, 15], stimulating continuous cell proliferation [16, 17] and arousing angiogenesis [18]. Finally, regular use of nonsteroidal anti-inflammatory drugs, such as aspirin, exhibits chemoprevention effect of CRC [19].

How the increased risk of CRC in IBD patients is mediated remains undefined. Nevertheless it is thought to involve genes linked to the inflammatory response, which result in the activation of neutrophils and the consequent generation of significant amounts of reactive oxygen species [20]. The continuous exposure of the gastrointestinal epithelium to reactive oxygen species is believed to be associated with an amplified mutation rate, which results in an increased likelihood of tumour development [20]. Thus, assuming that the underlying chronic inflammation in IBD is involved in the progression of CRC, genetic factors implicated in the chronic inflammatory process of IBD may simultaneously predispose patients without IBD to CRC.

There is strong evidence that *CARD15* mutations increase susceptibility to Crohn's disease (CD) [21–25]. Hence, the possible association of *CARD15* mutations with sporadic CRC has been studied recently in Polish, Greek, Finnish, New Zealand, Hungarian and German Caucasian CRC patients, but the results have been controversial (Table 1) [26–32]. Furthermore, almost all of these studies only assessed *CARD15* mutations in DNA extracted from blood or non-neoplastic tissue, i.e. involving only research of germline mutations [26, 27, 29–32]. Clarifying the potential involvement of *CARD15* somatic mutations in the pathogenesis of CRC also requires investigation in the neoplastic tissue. Therefore, at this stage, the role of *CARD15* variants in the development of CRC remains unclear. In light of these findings, and given that the frequency of *CARD15* mutations varies in different populations, our aim was to investigate whether germline and/or somatic *CARD15* mutations increased susceptibility to sporadic CRC in Portugal, a country with a high incidence and mortality associated with this disease. Additionally, we intended to investigate whether there are genotype–phenotype correlations in these patients.

Methods

Patients

One hundred and twelve consecutive Portuguese patients with CRC and without a previous diagnosis of IBD or any of the known hereditary cancer syndromes (male/female: 67/45; mean age at diagnosis of CRC 64.3±9.9 years old) and 152 healthy (cancer-free blood donors) sex-matched controls, drawn from the central region of Portugal, were genotyped for the three main *CARD15* mutations. Genomic DNA was isolated from whole blood in all the study participants and in 14 CRC patients it was also isolated from neoplastic biopsy specimens.

The vast majority of the group studied consisted of truly sporadic cases (95 patients, 84.8%), whilst 17 patients (15.2%) reported a history of CRC in at least one first-degree relative.

Comprehensive clinical data, including age and symptoms at diagnosis and the clinicopathologic characteristics of the tumour, were collected from each patient.

The research was approved by the local Ethics Committee and informed consent was obtained from all participants prior to enrolment into the study, in accordance with the Declaration of Helsinki.

Sample DNA extraction and genotyping

Genomic DNA was isolated from whole blood and biopsy specimens in accordance with the QIAamp Mini Kit (QIAGEN GmbH, Germany).

Table 1 *CARD15* and colorectal cancer: summary of association analyses

	Country [Reference]	Poland [26]		Greece [27]	Finnland [28, 29]	New Zealand [30]	Hungary [31]	Germany [32]	
		≤50years[a]	>50years[a]					Total	≤50years[a]
	CRC (*n*)	50	250	104	953/960/926[c]	133	194	1044	143
	Controls (*n*)	300	300	100	508/508/348[c]	201	200	724	724
R702W									
CRC	% Allele frequency	–	–	4.8	2.2	7.1	1.8	5.1	7.7
Control	% Allele frequency	–	–	1.0	2.1	3.0	1.5	4.6	4.6
	p value	–	–	0.02	0.88	0.03	0.78	0.5	0.03
G908R									
CRC	% Allele frequency	–	–	8.65	0.3	2.2	1.8	1.5	2.1
Control	% Allele frequency	–	–	3.5	0.2	0.8	1.8	1.2	1.2
	p value	–	–	0.025	0.57	0.09	0.95	0.43	0.36
3020insC									
CRC	% Allele frequency	2[b]	14.4[b]	12.5	1.9	2.2	3.6	3.6	3.8
Control	% Allele frequency	7[b]	7[b]	6	1.9	1.0	2.5	2.8	2.8
	p value	0.3010	0.0046	0.017	0.96	0.19	0.40	0.17	0.32
R702W/G908R/3020insC									
CRC	% Genotype frequency	–	–	51.9	–	21.8	14.4	10.2	25.2
Control	% Genotyple frequency	–	–	21.0	–	8.9	11.5	8.6	8.6
	p value	–	–	<0.0001	–	0.001	0.45	0.10	0.038

CRC colorectal cancer

[a] Age at diagnosis of CRC

[b] Genotype frequencies of carriership of mutant allele

[c] In the investigation of R702W, G908R and 3020insC were utilised 953, 960 and 926 CRC cases and 508, 508 and 348 controls, respectively

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 3020insC variant using SimpleProbe. The R702W variant was amplified and detected using the forward primer 5′-AgCCgCACAACCTTAgATCAC-3′, the reverse primer 5′-gCgggCACAggCATAgC-3′, the anchor probe 5′-LC Red640-gTCTggCACTCAgCCAgCAggCCCC-PH and the donor probe 5′-gCgCCAgAgCAgggCCTTCTCA-FL. For assay of the G908R mutation, 5′-gCACATATCAggTA CTCACTgACACT-3′ was used as the forward primer, 5′-TTA CCTgAgCCACCTCAAgC-3′ as the reverse primer, 5′-LC Red705-CTgAAAAggCCAAAAgAgTCAACAgAC-PH as the anchor probe and the 5′-CCACTCTgTTgCCCCAgAA-FL as the donor probe. In order to detect the 3020insC variant, PCR was carried out using the forward primer 5′-gACAgg TgggCTTCAgTAgA-3′, the reverse primer 5′-TgAggTTCggA gAgCTAAAACAg-3′ and the simple probe 5′-CTgCAggCCC CTTgAAAg-FLQ.

The reaction mixture consisted of 18 μl of the PCR master mix plus 2 μl of the DNA of each patient. The PCR master mix for the R702W variant included 5.0 mM $MgCl_2$, 1× LightCycler DNA master hybridization probes (Roche, Mannheim, Germany), 0.5 μM each primer and 0.1 μM each fluorescein and LC-Red-640-labelled probe. Cycling parameters consisted of one 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 fluorescent 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 $MgCl_2$, 1× LightCycler DNA master hybridization probes (Roche, Mannheim, Germany), 0.5 μM each primer and 0.2 μM each fluorescein and LC-Red-705-labelled probe. Cycling parameters consisted of one 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 originated by measuring the fluorescent 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 $MgCl_2$, 1× 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 fluorescent 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 (Tm) by plotting the negative derivative of the fluorescent signal corresponding to the temperature (−dF/dT) with the LightCycler software.

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

Statistical analysis

The significance of the differences in *CARD15* genotype frequencies between CRC patients and controls, as well as the association of *CARD15* variants with the clinico-pathologic characteristics, were assessed using χ^2 tests. Odds ratios (OR) were determined with the corresponding χ^2 distribution test and 95% confidence intervals (95% CI).

Survival and recurrence cumulative curves as a function of time were produced utilising the Kaplan–Meier approach and compared with the log-rank test.

Only p values <0.05 were considered significant.

Each genetic variant studied was in Hardy–Weinberg equilibrium.

The data was analysed using the Statistical Package for Social Sciences-SPSS (SPSS Inc., Chicago, Illinois, USA) computer software for Windows (version 17.0).

Results

The combined genotype frequency of R702W/G908R/3020insC was not statistically different between CRC patients and controls (16.1% vs. 9.9%, p=0.132; OR, 1.75; 95% CI, 0.84–3.64; Table 2). Individually, R702W was significantly more frequent in the CRC patients than the controls (12.5% vs. 5.3%, p=0.035; OR, 2.57; 95% CI, 1.04–6.36), whereas comparable genotypic frequencies of G908R and 3020insC were observed in both groups (Table 2). Among subjects with *CARD15* mutations, only one patient was homozygous for R702W, whilst all the others were heterozygous for a particular mutation (Table 2). No compound heterozygous carriers were identified.

In the 14 patients genotyped for *CARD15* mutations with DNA isolated from whole blood and also with DNA isolated from neoplastic biopsy specimens, complete genotypic agreement was found between the two samples, namely two patients heterozygous for R702W and the remaining 12 wild-type homozygous.

At least one variant of the *CARD15* was detected in ten (28.6%) of the 35 patients with CRC diagnosed under 60 years old and in eight (10.4%) of the 77 patients with ≥60 years old at diagnosis of CRC (p=0.015; OR, 3.45; 95% CI, 1.22–9.72; Table 3). Female CRC patients were more likely to carry a *CARD15* mutation than their male counterparts (24.4% vs. 10.4%, p=0.048; OR, 2.77; 95% CI, 0.98–7.82; Table 3). In contrast, patients having at least one first-degree relative diagnosed with CRC were no more likely to carry a *CARD15* mutation than patients

Table 2 Genotype frequencies of the *CARD15* variants in CRC patients and controls

Variant	Genotype			p	OR (95% CI)
	WT/WT	WT/MUT	MUT/MUT		
R702W					
CRC	98	13	1	0.035	2.57 (1.04–6.36)
Control	144	8	0		
G908R					
CRC	109	3	0	0.775	0.81 (0.19–3.46)
Control	147	5	0		
3020insC					
CRC	111	1	0	0.749	0.68 (0.06–7.55)
Control	150	2	0		
R702W/G908R/3020insC					
CRC	94	17	1	0.132	1.75 (0.84–3.64)
Control	137	15	0		

p value and respective OR (odds ratio) for differences in genotype frequencies between patients and controls

CRC colorectal cancer, *WT* wild-type, *MUT* mutant, *WT/WT* homozygous wild-type, *WT/MUT* heterozygous, *MUT/MUT* homozygous mutant, *OR* odds ratio, *CI* confidence interval

without this familial aggregation (11.8% vs. 16.8%, respectively; p=0.600; OR, 0.66; 95% CI, 0.14–3.17).

No associations were found between carriers of *CARD15* mutations and clinical manifestations, neither with tumour stage, size, differentiation or location (Table 3). Similarly, we did not find any correlation between *CARD15* mutations and survival or recurrence rates (Fig. 1).

In assessing genotype–phenotype correlations based only on the presence or absence of the *CARD15* R702W variant, we detected a statistical significance for the same correlations, namely a significantly higher incidence of mutation in patients with CRC diagnosed under 60 years old and in female patients (Table 4 and Fig. 2).

Discussion

IBD-related cancer serves as an excellent model of inflammation-associated cancer and might also provide many important clues to understanding the pathogenesis of sporadic CRC [11]. Thus, because the normal colon is arguably in a continual state of low-grade inflammation in response to its microbial flora, it is reasonable to speculate that, apart from IBD-associated CRC, sporadic CRC might be consequence of bacteria-induced inflammation [8].

Polymorphisms in *CARD15* have been associated with increased susceptibility to IBD, particularly to CD [21–25]. This discovery has led researchers to investigate the

Table 3 Clinicopathologic features of CRC patients with and without *CARD15* mutations

Variables	Total *n*	Carriers[a]n (%)	Non carriers *n*	*p*	OR (95% CI)
Gender					
Female	45	11 (24.4)	34	0.048	2.77 (0.98–7.82)
Male	67	7 (10.4)	60		
Age at diagnosis[b]					
<60 years	35	10 (28.6)	25	0.015	3.45 (1.22–9.72)
≥60 years	77	8 (10.4)	69		
Symptoms at diagnosis[c]					
Yes	97	16 (16.5)	81	0.756	1.28 (0.26–6.25)
No	15	2 (13.3)	13		
Tumour location					
Rectum	52	8 (15.4)	44	0.854 (rectum vs. left+right)	0.91 (0.33–2.51)
Left-colon	29	6 (20.7)	23	0.572 (rectum+left vs. right)	1.41 (0.43–4.67)
Right-colon	31	4 (12.9)	27		
Tumour size (mm)					
<30	28	3 (10.7)	25	0.373	0.55 (0,15–2.07)
≥30	84	15 (17.9)	69		
Differentiation					
Good	84	14 (16.7)	70	0.766 (good vs. moderate+poor)	1.20 (0.36–4.00)
Moderate	24	4 (16.7)	20	0.373 (good+moderate vs. poor)	–
Poor	4	0 (0)	4		
T stage					
1	16	3 (18.8)	13	0.753 (T1 vs. T2+T3+T4)	1.25 (0.32–4.91)
2	12	1 (8.3)	11	0.766 (T1+T2 vs. T3+T4)	0.83 (0.25–2.78)
3	66	12 (18.2)	54	0.532 (T1+T2+T3 vs. T4)	1.64 (0.34–7.85)
4	18	2 (11.1)	16		
N stage					
0	59	9 (15.2)	50	0.804	0.88 (0.32–2.41)
1 or 2	53	9 (17.0)	44		
M stage					
0	91	15 (16.5)	76	0.805	1.18 (0.31–4.53)
1	21	3 (14.3)	18		

CRC colorectal cancer, *OR* odds ratio; *CI* confidence interval

[a] Patients with *CARD15* R702W, G908R or 3020insC variants.

[b] Patients were divided into early (<60 years at diagnosis) and late (≥60 years at diagnosis) onset groups as in previous studies [27, 29–31], to allow for comparison.

[c] Symptoms: hematochezia, weight loss, anaemia, changes in bowel movement habits.

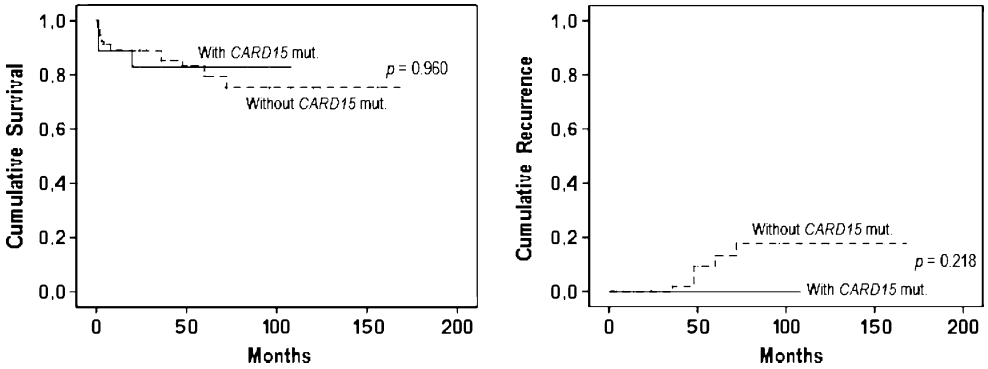

Fig. 1 Cumulative survival and recurrence rates in colorectal cancer patients with and without *CARD15* mutations

possible influence of this gene on the development of sporadic CRC, but the studies in this area have led to controversial results [26–32]. However, it has been demonstrated that there is regional heterogeneity within Europe in terms of the contribution of *CARD15* variants to CD susceptibility, reflecting the effects of differing founder populations, and we can suppose that the same phenomenon can be seen in CRC [21, 24, 25, 33–35]. Given this data and knowing the high CRC incidence and mortality rate in our country, this paper aimed to clarify whether germline and/or somatic *CARD15* mutations increase susceptibility to sporadic CRC in the Portuguese population and whether there are genotype–phenotype correlations in these patients.

We found that the frequency of R702W was significantly higher in the CRC patients than in the controls, whereas the frequency of G908R and 3020insC and the combined frequencies of the three key *CARD15* mutations did not differ significantly between the two groups. These results agree with the findings of Roberts et al. in the New Zealand population where, in assessing the effect of each variant individually in a series of 133 CRC patients, only a significant association between R702W and CRC was detected (OR=2.30; 95% CI, 1.1–5.0) [30]. However, in the New Zealand study, contrarily to our work, evidence for the association of the combined frequency of the three common *CARD15* variants with the risk of CRC was obtained (OR=2.8; 95% CI, 1.5–5.4) [30]. Moreover, Papaconstantinou et al. [27] found that all the three major *CARD15* mutations were significantly elevated in Greek CRC patients (OR=4.06; 95% CI, 2.19–7.52), in Poland Kurzawski et al. [26] described an association between the *CARD15* 3020insC variant and CRC in patients aged over 50 at the time of diagnosis (OR=2.23; 95% CI, 1.23–4.10) and recently a study conducted by Möckelmann et al. [32] found a significant association between *CARD15* mutations and CRC susceptibility in a German cohort of patients aged under 50 (p=0.038). In contrast, Alhopuro et al. and Tuupanen et al. found no link between CRC and any of the three main *CARD15* mutations in a population-based series of 1042 Finnish CRC patients, although it must be borne in mind that the background frequency of *CARD15* variants in the Scandinavian population is much lower [28, 29]. Likewise, Lakatos et al. observed similar variant allele frequencies in both the patient and control Hungarian groups [31].

As in CD, where *CARD15* mutations are associated with the early onset of disease, ileal localization and stricturing phenotype, it is possible that these mutations may also be predictive of the age of CRC presentation and of tumour behaviour [36–41]. Associations between *CARD15* variants and specific clinicopathologic characteristics were observed in the Polish, Greek, New Zealand and German CRC cohorts [26, 27, 30, 32]. Within the Polish CRC cohort, the frequency of the 3020insC variant was found to be significantly elevated in patients aged over 50 at the time of diagnosis [26]. In the Greek cohort, the *CARD15* variant carriers had more frequently advanced stage tumours [27].

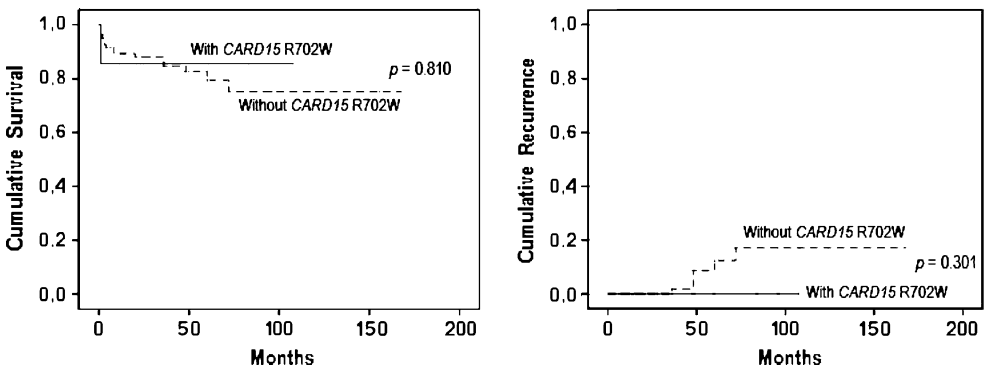

Fig. 2 Cumulative survival and recurrence rates in colorectal cancer patients with and without *CARD15* R702W variant

Table 4 Clinicopathologic features of CRC patients with and without *CARD15* R702W variant

Variables	Total *n*	Carriers[a] n (%)	Noncarriers *n*	*p*	OR (95% CI)
Gender					
Female	45	9 (20.0)	36	0.049	3.10 (0.96–9.97)
Male	67	5 (7.5)	62		
Age at diagnosis[b]					
<60 years	35	9 (25.7)	26	0.004	4.99 (1.53–16.25)
≥60 years	77	5 (6.5)	72		
Symptoms at diagnosis[c]					
Yes	97	12 (12.4)	85	0.916	0.92 (0.18–4.58)
No	15	2 (13.3)	13		
Tumour location					
Rectum	52	4 (7.7)	48	0.152 (rectum vs. left+right)	0.42 (0.12–1.42)
Left-colon	29	6 (20.7)	23	0.936 (rectum+left vs. right)	0.95 (0.28–3.29)
Right-colon	31	4 (12.9)	27		
Tumour size (mm)					
<30	28	2 (7.1)	26	0.322	0.46 (0.10–2.20)
≥30	84	12 (14.3)	72		
Diferentiation					
Good	84	11 (13.1)	73	0.741 (good vs. moderate+poor)	1.26 (0.32–4.87)
Moderate	24	3 (12.5)	21	0.441 (good+moderate vs. poor)	–
Poor	4	0 (0)	4		
T stage					
1	16	1 (6.3)	15	0.414 (T1 vs. T2+T3+T4)	0.43 (0.05–3.50)
2	12	0 (0)	12	0.099 (T1+T2 vs. T3+T4)	0.20 (0.03–1,62)
3	66	11 (16.7)	55	0.846 (T1+T2+T3 vs. T4)	1.17 (0.24–5.74)
4	18	2 (11.1)	16		
N stage					
0	59	8 (13.6)	51	0.721	1.23 (0.40–3.81)
1 or 2	53	6 (11.3)	47		
M stage					
0	91	12 (13.2)	79	0.647	1.44 (0.30–7.00)
1	21	2 (9.5)	19		

CRC colorectal cancer, *OR* odds ratio, *CI* confidence interval

[a] Patients with *CARD15* R702W variant.

[b] Patients were divided into early (<60 years at diagnosis) and late (≥60 years at diagnosis) onset groups as in previous studies [27, 29–31], to allow for comparison

[c] Symptoms: hematochezia, weight loss, anaemia, changes in bowel movement habits.

In the New Zealand study, female CRC patients were far less likely to carry a *CARD15* mutation than their male counterparts [30]. Finally, in the German cohort, the frequency of R702W and the combined frequency of genotypes harbouring R702W, G908R or 3020insC were found to be significantly higher in patients ≤50 years old at the time of the CRC diagnosis [32]. In our CRC cohort we found a significantly higher incidence of *CARD15* mutations in patients diagnosed under 60 years old and in female patients, and we did not detect any differences in tumour behaviour between individuals who carried *CARD15* mutations and those who had a wild-type *CARD15* genotype. These data suggest that also with regard to genotype–phenotype correlations there is regional heterogeneity.

As in other studies, we found no association between *CARD15* mutations and a familial aggregation of CRC [26, 28, 29].

In order to assess the possible contribution of loss of the wild-type *CARD15* allele in 3020insC-related tumourigenesis, Alhopuro et al. sequenced the tumour tissue DNA of all the patients heterozygous for the *CARD15* 3020insC variant in normal tissue DNA [28]. They found loss of heterozygosity in only one of the 33 heterozygous patients and concluded that there was no evidence of contribution of loss of the wild-type *CARD15* allele in the

CRC carcinogenesis [28]. These results do not allow any conclusions to be drawn as to whether *CARD15* somatic mutations are involved in the predisposition to CRC. This assessment also implies the genotyping of tumour tissue DNA in patients without C*ARD15* mutations in non-neoplastic DNA. In our work, the entire *CARD15* genotypic agreement between blood and neoplastic samples suggests that CRC susceptibility associated with *CARD15* variants is linked to germline mutations without the apparent participation of somatic mutations. To our knowledge, this is the only study involving research of *CARD15* variants in both neoplastic and non-neoplastic DNA within a random group of CRC patients. Thus, it is also the first study suggesting that only *CARD15* germline mutations are involved in the increased susceptibility to CRC associated with this gene. This important and interesting topic needs further confirmation in different and larger samples of CRC patients.

The potential limitations of our study should be mentioned. Firstly, the size of the study population is relatively small. Secondly, as *CARD15* is associated with immune modulation, we cannot rule out the fact that other genetic factors involved in the immune response or implicated in the control of inflammatory response may also contribute to the risk of disease. Thirdly, the potential effect of environmental factors and/or their interaction with *CARD15* mutations have not been evaluated. Finally, this work was performed in a teaching and referral hospital and therefore our results may not be applicable to institutions with different patient populations.

Our findings suggest that genetic variability within *CARD15* may be one of the factors contributing to the elevated risk of CRC observed in the Western world, and particularly in Portugal. However, the available results show regional heterogeneity in terms of the contribution of *CARD15* variants to CRC susceptibility. Moreover, environmental factors and additional genetic factors vary greatly between populations, and genetic polymorphisms are thought to play a role in determining how individuals respond at cellular level to various environmental factors [42]. Thus, further data from different and larger populations is needed to determine whether *CARD15* mutations lead to a predisposition to CRC and, if so, to identify the additional determinants necessary for this increased susceptibility. A thorough investigation of these issues may improve screening recommendations, could shed light on the mechanisms of sporadic malignancy initiation and progression and might be critical to the development of pharmaceutical agents for cancer chemoprevention. Additionally, further research is needed in order to clarify the potential role of *CARD15* mutations in cancer pathogenesis and pathophysiology.

In conclusion, our study suggests that the *CARD15* R702W variant might be a predisposing factor to sporadic CRC in the Portuguese population, particularly for those aged under 60 and in female patients. Additionally, this is the first study to hint that this susceptibility to CRC is linked only to germline *CARD15* mutations**.** Nevertheless, *CARD15* mutations do not appear to influence the pathological expression of CRC in Portuguese patients. Regional heterogeneity within the *CARD15* genotype in CRC patients shows the importance of the genetic assessment and evaluation of its correlation with the phenotype in different populations. Additional research, using larger patient groups as well as other populations, is required in order to unequivocally determine the role of *CARD15* variants in CRC risk and carcinogenesis.

Conflicts of interest The authors declare that they have no conflict of interest.

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