Genetics and tumor genomics in familial colorectal cancer

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Chapter 4

Enrichment of Low Penetrance Susceptibility Loci in a Dutch Familial Colorectal Cancer Cohort

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Enrichment of Low Penetrance Susceptibility Loci in a Dutch Familial Colorectal Cancer Cohort

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Abstract

Recent genome-wide association studies have identified several loci that confer an increased risk of colorectal cancer (CRC). We studied the role of the 8q24.21 (rs6983267), 18q21.1 (rs12953717), 15q13.3 (rs4779584), 11q23.1 (rs3802842), 8q23.3 (rs16892766), and 10p14 (rs10795668) risk variants in a series of 995 Dutch CRC cases and 1340 controls. The CRC cases were selected on basis of having a family history of CRC and/or early-onset disease. The detailed clinical and molecular data available on the cases allowed us to examine the relationship between risk variants and clinicopathologic characteristics. We replicated the association with an increased risk of CRC cancer for all loci, except 10p14. The association with the variant on chromosome 15q13.3 was confirmed for the first time. The risks associated with variants in our series were higher (not significant) than those previously reported, consistent with our series reflecting genetic enrichment. Moreover, we show that familial CRC cases possess an increased number of risk alleles compared with solitary CRC cases (early-onset; mean age at diagnosis of 48.5 years). We also identified a significant increase in the number of risk alleles in families with early-onset disease (≤50 years) compared with late-onset families (>50 years). In solitary CRC patients, enrichment for risk alleles was not observed, suggesting that other causes of increased CRC risk play a role in these cases. Overall, our results suggest that clustering of low-risk variants may explain part of the excess risk in CRC families. (Cancer Epidemiol Biomarkers Prev 2009;18(11):3062–7)

Introduction

Around a third of all colorectal cancer (CRC) has been shown to be attributable to heritable factors (1). High Penetrance mutations, such as those in the mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) causing the Lynch syndrome, *APC* in Familial Adenomatous Polyposis, and *MUTYH* in *MUTYH*-associated polyposis, only account for ~5% of CRC (2-4). Although the underlying basis of the residual excess familial risk is presently undefined, it is likely that coinheritance of several common alleles each conferring a low CRC risk contribute to this excess familial risk.

Recent genome-wide association studies have vindicated this hypothesis and several loci have been robustly shown to be associated with an increased risk of developing CRC. The first of these was a variant at 8q24.21 defined by the single nucleotide polymorphism (SNP) rs6983267 (5-8). This variant was also shown to be associated with increased risks of both prostate and ovarian cancer (9, 10). Recently, this locus has also been associated with the development of multiple colorectal adenomas (11). Three SNPs on 18q21.1 were reported to be associated with an increased CRC risk. These three variants (rs4939827, rs12953717, and rs4464148) map to an intronic sequence of *SMAD7*, an antagonist of transforming growth factor-β signaling (12, 13). A third locus associated with an increased CRC risk was identified on chromosome 15q13.3 (14). Previously, linkage of this locus to CRC was reported in three Ashkenazi families (15, 16). The strongest association for this locus was reported with rs4779584, close to the genes *SCG5*, *GREM1*, and *FMN1*. A fourth association was identified in a gene-rich region on 11q23 with SNP rs3802842 (13, 17). Furthermore, SNP rs10795668 (located at 10p14) and SNP rs16892766 (located at 8q23.3) were reported to be associated with an increased risk of CRC (18). This latter variant at 8q23.3 is linked to *EIF3H*, a translation initiation factor. Current data suggest that each of these loci act independently in a dose-dependent manner with those individuals possessing multiple risk variants having relatively substantive risks over those carrying few risk alleles (17).

Association of these six risk loci with several clinical or pathologic parameters has been described. For rs3802842 (11q23) and rs4939827 (18q21.1), the risk of developing rectal cancer was found to be greater than the risk of

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Table 1. Scoring system for family characteristics

Category	Description
I	Amsterdam Criteria II positive families*
II	At least two FDR affected with CRC, at least one diagnosed ≤50 y in one or two generations
III	Solitary CRC patient
IV	At least three FDR affected with CRC, diagnosed >50 y in one or two generations
V	Two FDR affected with CRC, diagnosed >50 y in one or two generations

Abbreviation: FDR, first-degree relative.
*Including largely non-Lynch or so-called Lindor Type X families (29).

developing colonic cancer (13). Moreover, Tomlinson et al. (18) reported that the association with rs10795668 (10p14) was stronger for rectal cancer than for colonic tumors. The association of the locus at 8q24.21 was described to be stronger for patients under the age of 50 years than for those 50 years of age or older (8). Similarly, the effect of rs16892766 (8q23.3) on CRC risk has also been described to be stronger in younger individuals (<60 years; ref. 18). Finally, Tuupanen et al. (19) identified a tendency for association of rs6983267 (8q24.21) with microsatellite stable (MSS) cancer and a family history of extracolonic cancers.

The effect of the variants at these six loci has also been studied in Lynch syndrome patients, carrying a mutation in one of the mismatch repair genes. In Lynch patients, rs16892766 (8q23.3) is associated with an elevated CRC risk. For rs3802842 (11q23.1), an elevated risk of developing CRC was described for female Lynch patients only (20).

To further inform on the relationship of variants at these six loci with the development of CRC, we have genotyped 8q24.21, 18q21.1, 15q13.3, 8q23.3, 11q23.1, and 10p14 variants in a cohort of Dutch CRC cases, enriched for a positive family history and/or an early onset of disease. Detailed clinical and pathologic data on the cases has allowed us to examine the relationship between clinicopathology and genotype in the setting of familial disease.

Materials and Methods

Study Population. We studied 995 index cases, all of which were diagnosed with CRC (48% male, 52% female), and 1340 controls (47% male, 53% female). These cases fulfill the clinical criteria for microsatellite instability (MSI) testing, installed in the Netherlands since 1997 and formalized in 2008, based on early onset of the disease and/or familial clustering of CRC (Supplementary Table S1). Most cases were sent in through the clinical genetics department. Only one case per family was included in our cohort. Samples were collected mostly in a period from 1997 to 2007. The samples largely originate from the southwestern part of the Netherlands. Eighty-two percent of all tested tumors were MSS, whereas 7% had a low level of MSI (MSI-L), and 12% were MSI-H. Of the MSI-H tumors, 93% were MSI-H on a sporadic basis. Overall, the cohort includes 10 Lynch syndrome patients.

Controls were 894 healthy blood donors from the southwest region of the Netherlands. Additional 446 controls were derived from individuals that presented at the Laboratory for Diagnostic Genome Analysis at the Leiden University Medical Centre for presymptomatic testing for noncancer syndromes and tested mutation-negative for these syndromes. Age was not known for all controls. Therefore, the control cohort might include some young individuals that develop CRC later in life. This would reduce the power of our analysis and therefore could have resulted in an underestimation of the associations.

We genotyped the following SNPs in the CRC cases and the controls: rs16892766, rs6983267, rs10795668, rs3802842, rs4779584, and rs12953717.

The study was approved by the local Medical Ethical Committee (protocol P01.019). Samples were handled according to the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences.[8]

DNA Isolation. DNA was extracted from either peripheral blood or from formalin-fixed paraffin embedded (FFPE) normal tissue. DNA was extracted from blood samples using an automated procedure (Gentra Systems). DNA from FFPE tissue was isolated using xylol, ethanol, and overnight incubation at 56°C in 120 μL of PK1 lysis buffer and 5 μL Proteinase K. The suspension was then incubated for 10 min at 100°C and centrifuged for 10 min at 13,000 rpm. The supernatant containing the DNA was carefully transferred to a new tube. DNA was quantified using the picogreen method (Invitrogen-Molecular Probes).

MSI Analysis. Eight microsatellite markers were evaluated to determine the MSI status of the tumors. As recommended by the National Cancer Institute Workshop on MSI for Cancer Detection and Familial Predisposition, two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250) were analyzed, supplemented with three mononucleotide repeat markers (BAT40, MSH3, and MSH6; ref. 21). Tumors were classified as MSS when no instability was seen for any of the eight markers. When instability was seen in <30% of the markers, tumors were classified as MSI-L. When >30% of the markers showed instability, the tumors were classified as MSI-H.

Genotyping. Genotyping of the variants rs12953717, rs3802842, rs6983267, and rs16892766 was done using the KASPar method following the manufacturer's protocol (KBioscience). PCR, with two allele-specific forward primers and one common reverse primer, was done in a GeneAmp PCR system 9700 (Applied Biosystems). Detection was done in an ABI PRISM 7900HT (Applied Biosystems).

Genotyping of rs10795668 was done using a TaqMan SNP Genotyping Assay for samples that were derived from FFPE tissue according to the manufacturer's protocol (Applied Biosystems). The leukocyte DNA samples were genotyped using high-resolution melting curve analysis. Primers for high-resolution melting curve analysis were designed using Primer3. In short, 5 μL PCR reactions were analyzed on a LightCycler (Roche) using the LightCycler software (version 1.5.0, Roche). PCR reactions consisted of 10 ng DNA, iQ Supermix (Bio-Rad), 1 μmol/L SYTO9 (Invitrogen), and 2 pmol primers.

For rs4779584, all leukocyte DNA was genotyped using the KASPar method, whereas the high-resolution melting

[8] http://www.federa.org

Table 2. Characteristics of the study population

	Cases	Controls
Gender		
Male	453 (48%)	633 (47%)
Female	492 (52%)	707 (53%)
Age at diagnosis		
≤50 y	291 (42%)	
>50 y	395 (58%)	
MSI status		
MSI-L/MSI-H	134 (18%)	
MSS	596 (82%)	
Tumor location		
Right sided	218 (33%)	
Left sided	438 (67%)	
Tumor stage		
A	27 (5%)	
B	301 (51%)	
C	204 (35%)	
D	55 (9%)	
Polyps		
No	245 (32%)	
Yes	474 (63%)	
Unknown	38 (5%)	
Family characteristics		
Category I	66 (13%)	
Category II	105 (21%)	
Category III	144 (28%)	
Category IV	102 (20%)	
Category V	66 (13%)	
Other	23 (5%)	

curve analysis method was applied to the samples isolated from FFPE tissue. Samples that failed to produce a genotype were reanalyzed using the TaqMan SNP genotyping assay (Applied Biosystems). Primer details are available upon request.

To check the quality of the genotyping, >5% of the samples were analyzed in duplicate. The concordance was >98%. All SNP genotype frequencies in our cohort fit the Hardy-Weinberg equilibrium as assessed using a χ^2 test.

To facilitate data management and analysis, we developed a relational database that is based on MS Access and structured query language.

Clinicopathologic Characteristics. For all cases, information was available for several clinical genetic and pathologic parameters. This information included age at diagnosis, microsatellite status, tumor grade, location of the tumor (left of the flexura lienalis versus right of the flexura lienalis), additional development of adenomas, and family history characteristics. Information on the presence of adenomas was derived from Pathological Anatomical District Automated Archives, a nationwide network and registry of histopathology and cytopathology in the Netherlands (22). The latter was scored with the scheme displayed in Table 1.

Statistical Analysis. Genotype frequencies in the CRC cases and controls were tested for deviations from expected frequencies under Hardy-Weinberg Equilibrium using a χ^2 test using a significance level of 5% (one degree of freedom). Odds ratios (OR), including their 95% confidence intervals (CI), were calculated using logistic regression analysis. Bonferroni's correction for multiple testing was applied. Associations between CRC risk loci and clinical and pathologic parameters were assessed using a χ^2 test (one degree of freedom) in CRC cases only.

Power calculation was done under the assumption of an additive model and an α of 0.05.

We used a Cochran-Armitage test of trend using ordered categorical data to study the effect of possessing an increased number of risk alleles (counting one for heterozygotes and two for homozygotes). The most common number of risk alleles was used as a reference to calculate ORs. These statistical analyses were done in SPSS 16.0.

Pairwise interactions between the SNPs were studied using Plink (v1.05; ref. 23).

Results and Discussion

We studied the effects of rs16892766 (8q23.3), rs6983267 (8q24.21), rs10795668 (10p14), rs3802842 (11q23.1), rs4779584 (15q13.3), and rs12953717 (18q21.1) in a Dutch familial CRC cohort. This cohort is enriched for a positive family history for CRC and/or early onset of disease. The main characteristics of the study cohort are detailed in Table 2.

Association Analysis. Variant rs6983267 (8q24.21) was significantly associated with an increased risk of CRC in our familial cohort, with an allelic OR of 1.29 (95% CI, 1.14-1.49; $P = 7.2 \times 10^{-5}$; Table 3). We also replicated the association of variant rs12953717 (18q21.1) with CRC risk in our cohort, with an allelic OR of 1.23 (95% CI, 1.09-1.38; $P = 8.6 \times 10^{-4}$). Curtin et al. (24) also described associations

Table 3. Association between CRC risk and low-risk variants

SNP	Chromosome region	Minor allele*	Allele frequencies†		OR (95% CI) Allelic	*P*	GWAS OR (95% CI) Allelic	Power
			Cases	Controls				
rs6983267	8q24.21	G	0.58	0.52	1.29 (1.14-1.46)	7.2×10^{-5}‡	1.24 (1.17-1.33)§	95%
rs12953717	18q21.1	T	0.45	0.40	1.23 (1.09-1.39)	8.6×10^{-4}‡	1.17 (1.12-1.22)‖	75%
rs4779584	15q13.3	T	0.24	0.18	1.45 (1.24-1.69)	3.8×10^{-6}‡	1.23 (1.14-1.34)§	81%
rs3802842	11q23.1	C	0.31	0.27	1.26 (1.11-1.43)	4.4×10^{-4}‡	1.11 (1.08-1.15)¶	38%
rs10795668	10p14	A	0.31	0.31	1.02 (0.88-1.17)	0.84	0.89 (0.86-0.91)§	45%
rs16892766	8q23.3	C	0.10	0.09	1.23 (1.00-1.50)	0.05	1.25 (1.19-1.32)§	53%

NOTE: GWAS OR, OR identified in previous genome-wide association studies. Power was calculated assuming an additive model and an α of 0.05.
*According to the HapMap CEU frequencies.
†In our cohort.
‡Associations remained significant after correction for multiple testing.
§Tomlinson et al., 2008.
‖Jaeger et al., 2008.
¶Tenesa et al., 2008.

Table 4. ORs for cumulative number of risk alleles

No. risk alleles	No. cases (%)	No. controls (%)	OR (95%CI)	*P*
0	13 (1.8)	18 (2.9)	0.65 (0.31-1.36)	0.25
1	47 (6.7)	67 (10.7)	0.63 (0.41-0.96)	0.03
2	135 (19.2)	157 (25.0)	0.77 (0.57-1.04)	0.09
3	197 (28.0)	176 (28.0)	1.00 (reference)	
4	162 (23.0)	132 (21.0)	1.10 (0.81-1.49)	0.56
5	102 (14.5)	60 (9.6)	1.52 (1.04-2.22)	0.03
≥6	47 (6.7)	18 (2.9)	2.33 (1.31-4.17)	4.2×10^{-3}
Total	703 (100)	628 (100)		$P_{Trend} = 1.1 \times 10^{-7}$

NOTE: The results shown in this table are illustrated in Fig. 1.

between the risk alleles on 8q24.21 and 18q21.1 and CRC risk in a cohort that included familial CRC. For rs4779584 (15q13.3), a strong association with CRC risk was identified, with an allelic OR of 1.45 (95% CI, 1.22-1.67; $P = 3.8 \times 10^{-6}$). To the best of our knowledge, these results provide the first replication of the association between the locus at 15q13.3 and CRC risk. A strong association was also identified for rs3802842 on chromosome 11q23.1 (allelic OR, 1.26; 95% CI, 1.11-1.44; $P = 4.4 \times 10^{-4}$). The results for rs3802842 have previously been reported for 783 of 995 samples (17). An allelic OR of 1.23 for rs16892766 (8q23.3) was found in our cohort (95% CI, 1.00-1.51; $P = 0.05$). This association does not remain significant after correction for multiple testing. The results for rs16892766 (8q23.3) have previously been reported for 783 of 953 of the samples (18). Overall, the ORs identified in our cohort tend to be increased compared with the ORs described in the initial genome-wide association studies, consistent with our series reflecting genetic enrichment (Table 3). Although the ORs are consistently increased, the CIs overlap, indicating that the differences are not statistically significant.

Unlike the previous loci, we were unable to replicate the association between rs10795668 (10p14) and CRC risk. In our cohort, the locus on 10p14 was not associated with an increased risk of CRC (allelic OR, 1.02; 95% CI, 0.88-1.17; $P = 0.84$). The absence of an association between this locus and CRC risk may be explained by a lack of power to detect the association in our study, although the power to detect association with a minor allele frequency of 0.33 and an OR of 0.89 was 45% in our study, and association with rs3802842 (11q23.1) was detected with a prior power of 38%. A second explanation could be a difference between the Dutch population compared with the English population. Of note, this locus on chromosome 10p14 was also not captured by meta-analysis of two large genome-wide association studies (25).

Clinicopathologic Characteristics. We studied the association of the six risk loci mentioned above with several clinicopathologic parameters including the following: gender, age at diagnosis, polyp development, tumor stage, family characteristics (Table 1), tumor location, and MSI status. In our cohort, the association between rs12953717 (18q21.1) and CRC risk is stronger in cases with left-sided cancer (OR, 1.43; 95% CI, 1.22-1.68) compared with cases with right-sided cancer, where the association could not be detected (OR, 1.02; 95% CI, 0.82-1.27; $P = 0.03$). Further analysis shows that the association of rs12953717 and CRC is, among the left-sided cancers, stronger for the tumors located at the rectosigmoid junction or in the rectum (OR, 1.58; 95% CI, 1.28-1.95) compared with left-sided tumors proximal of the rectosigmoid junction (OR, 1.30; 95% CI, 1.04-1.61; $P = 0.04$). Similarly, a stronger effect of rs12953717 on CRC risk was seen for familial CRC cases with at least two first-degree relatives affected with CRC (OR, 1.51; 95% CI, 1.25-1.83) compared with solitary CRC cases (early-onset; mean age at diagnosis of 48.5 years; OR, 0.86; 95% CI, 0.65-1.14; $P = 8.6 \times 10^{-4}$). No relationship was identified between tumor location and family characteristics ($P = 0.16$), indicating that these two parameters are independently associated with rs12953717. No statistically significant associations between rs12953717 (18q21.1) and the other clinicopathologic parameters (including gender, age at diagnosis, polyp development, tumor stage, and MSI status) were found. For all other loci, no significant associations were found between the variants and gender, age at diagnosis, tumor location, family characteristics, polyp development, tumor stage, or MSI status. However, ORs for rs12953717 (18q21.1),

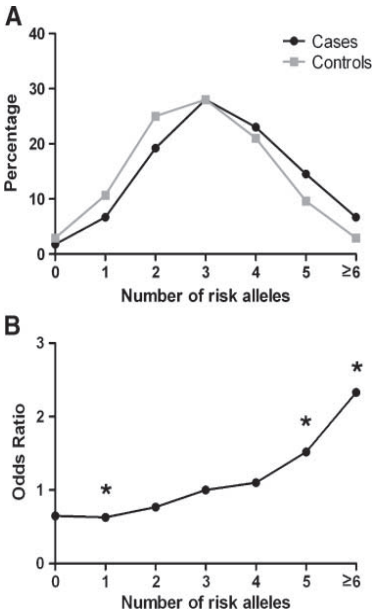

Figure 1. Number of risk alleles in the CRC cases and the controls. **A.** The distribution of the number of risk alleles in the CRC cases and the controls. **B.** The OR for each category. The most common number of risk alleles (three) was set as a reference. *, $P < 0.05$.

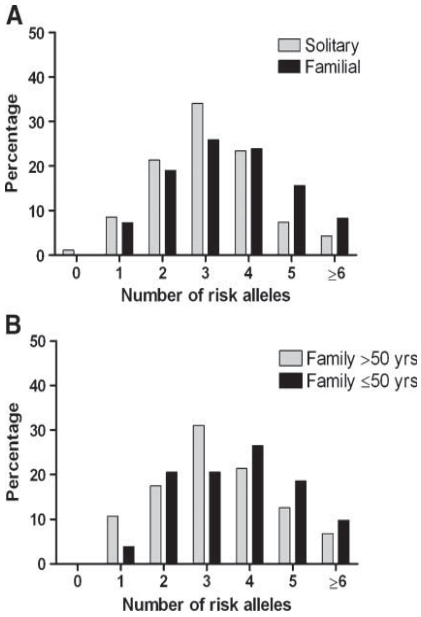

Figure 2. Relationship between number of risk alleles and family characteristics. **A.** The distribution of the number of risk alleles per individual in solitary cases and familial cases (with at least two first-degree relatives affected with CRC). **B.** The distribution of risk alleles in families with an age at diagnosis 50 y or younger and families with an age at diagnosis older than 50 y of age. In both graphs, the distributions differ significantly from each other (**A**, $P_{Trend} = 0.03$; **B**, $P_{Trend} = 0.04$).

rs4779584 (15q13.3), and rs16892766 (8q23.3) were increased in MSS cases compared with MSI-H cases, although these differences were not significant. These results suggest that the role of low-risk variants play in MMR-deficient tumors is reduced compared with MMR-proficient tumors, despite the sporadic nature of the MSI-H cases in our cohort. And although rs16892766 (8q23.3) is significantly associated with an increased CRC risk in MMR mutation carriers (20), its role in MMR-deficient tumors with a sporadic nature seems reduced. Several associations between the risk loci and clinicopathologic parameters have previously been described, including associations between rectal cancer and 11q23.1 and 18q21.1, between early disease onset and both 8q24.21 and 8q23.3, and between MSS tumors and 8q24.21 (8, 13, 18, 19). However, we were unable to replicate any of these associations in our cohort.

Risk Allele Distribution. We investigated the effect of possessing an increased number of risk alleles on CRC risk (Table 4). We counted the number of risk alleles per individual and then compared the distribution in CRC cases versus controls. We identified a significant increase in the number of risk alleles in CRC cases compared with controls ($P_{Trend} = 1.1 \times 10^{-7}$). Moreover, there was a gradual increase in the OR with an increased number of risk alleles, although not all increases in the OR were significant. For individuals possessing six or more risk alleles, an OR of 2.3 (95% CI, 1.31-4.17) was observed. These results are illustrated in Fig. 1. Our findings are in line with previous reports on the cumulative effect of these risk alleles on CRC risk (17). Similar cumulative effects have been described for other cancers, including prostate cancer and head and neck cancer (26-28).

Remarkably, a significant difference in the number of risk alleles was observed between solitary cases (early-onset; mean age at diagnosis of 48.5 years) and familial CRC cases (at least two affected first-degree relatives). The familial CRC cases had significantly more risk alleles compared with solitary CRC cases ($P_{Trend} = 0.03$), suggesting that low-risk variants indeed cluster in families affected with CRC (Fig. 2). In addition, the latter finding suggests that other genetic models (such as a recessive origin) might play a role in solitary CRC cases. Additionally, we found that families with an early onset of the disease (≤50 years of age) had significantly more risk alleles ($P_{Trend} = 0.04$) than families with a late onset of the disease (>50 years of age). These results suggest that possessing an increasing number of risk alleles decreases the age of onset of the disease. However, similar relationship between the number of risk alleles and disease onset was not seen at the individual level.

Analysis of pairwise interactions did not yield evidence for an interaction between any of the risk alleles. These results are consistent with the analyses in a recent meta-analysis, where all comparisons were based on at least 13,000 individuals (25).

In conclusion, we replicated the association between CRC risk and loci on chromosomes 8q23.3, 8q24.21, 11q23.1, 15q13.3, and 18q21.1. This is the first study to replicate the association between CRC risk and the locus on 15q13.3, although the association with a locus on chromosome 10p14 could not be replicated in our cohort. The ORs for rs6983267 (8q24.21), rs12953717 (18q21.1), rs4779584 (15q13.3), and rs3802842 (11q23.1) tend to be higher than those seen in the genome-wide analysis, possibly reflecting enrichment for positive family history of CRC in our cohort. Moreover, we saw enrichment in the number of risk alleles in patients with at least two first-degree family members affected with CRC compared with solitary CRC patients. Similar enrichment was identified in families with early-onset disease (≤50 years of age) compared with families with late-onset of CRC (>50 years of age). Overall, our results suggest clustering of low-risk variants in familial CRC, which is likely to contribute to the observed excess risk in relatives of patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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