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Original Paper

An Intronic Germline Transition in the HNPCC Gene *hMSH2* is Associated with Sporadic Colorectal Cancer

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The aim of this study was to determine whether an intronic germline substitution in the hereditary non-polyposis colorectal cancer (HNPCC) gene *hMSH2* represents a genetic risk factor for sporadic CRC. Possible effects of this substitution were investigated by assessment of microsatellite instability and *hMSH2* cDNA sequencing. Constitutional DNA from patients with sporadic CRC and healthy controls from the same region in Germany was analysed for the intronic germline T→C transition six bases upstream of exon 13 of *hMSH2*. 29 of 106 patients (27%) were found to harbour the germline T→C transition as opposed to only 13 of 125 controls (10%; $P < 0.001$; OR 3.2, CI 1.58–6.63). CRCs from patients with the substitution displayed neither clinical HNPCC-like features nor an increased rate of microsatellite instability. No abnormal cDNA sequence was found at the exon 12–13 border. These data suggest a 3.2-fold increased risk of sporadic CRC for individuals with the intronic *hMSH2* transition. However, this substitution might not be pathogenic itself, but may be linked to a locus nearby that is. © **1997 Elsevier Science Ltd.**

Key words: colorectal cancer, hereditary, *hMSH2*, human, transition, microsatellite instability, splicing

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INTRODUCTION

COLORECTAL CANCER (CRC) affects approximately 1 out of 16 individuals in Western industrialised countries [1]. Only 5–10% of CRC cases are caused by highly penetrant germline mutations in known genes such as the human *MSH2* gene on chromosome 2p [2–4]. *hMSH2* belongs to a group of at least four DNA mismatch repair genes, which, if mutated, cause hereditary non-polyposis colorectal cancer (HNPCC), a tumour syndrome with a well-known tendency to include extracolonic organs [5]. CRCs from HNPCC patients display characteristic clinical features (young age at diagnosis, preferential localisation in the proximal colon, positive family history of CRC or other HNPCC-associated tumours), as well as alterations of microsatellite DNA sequences [5–8]. These alterations in tumour DNA are denoted as microsatellite instability (MIN), which results from deficient DNA mismatch repair [9]. However, for the majority of sporadic CRCs (approximately 90–95%), few predisposing genetic alterations are known [10, 11]. Incompletely penetrant germline mutations might enhance susceptibility to CRC only moderately, resulting in apparently sporadic cases [2, 12]. Recently, an intronic germline substitution in *hMSH2* has been shown to increase the risk of CRC in patients with ulcerative colitis [13]. Accordingly, the goal of our study was to investigate whether this substitution is also associated with sporadic CRC. Because the substitution is located near the splice acceptor site at the intron–exon border, it might become functional by inducing aberrant splicing of *hMSH2* mRNA [3, 13, 14]. In this case, an HNPCC-like phenotype and marked MIN are to be expected [15]. Therefore, CRCs from patients with and without the substitution were compared for clinical HNPCC features and MIN. For detection of aberrant splicing *hMSH2* cDNA was generated, and its exon 12–13 border was investigated by sequence analysis.

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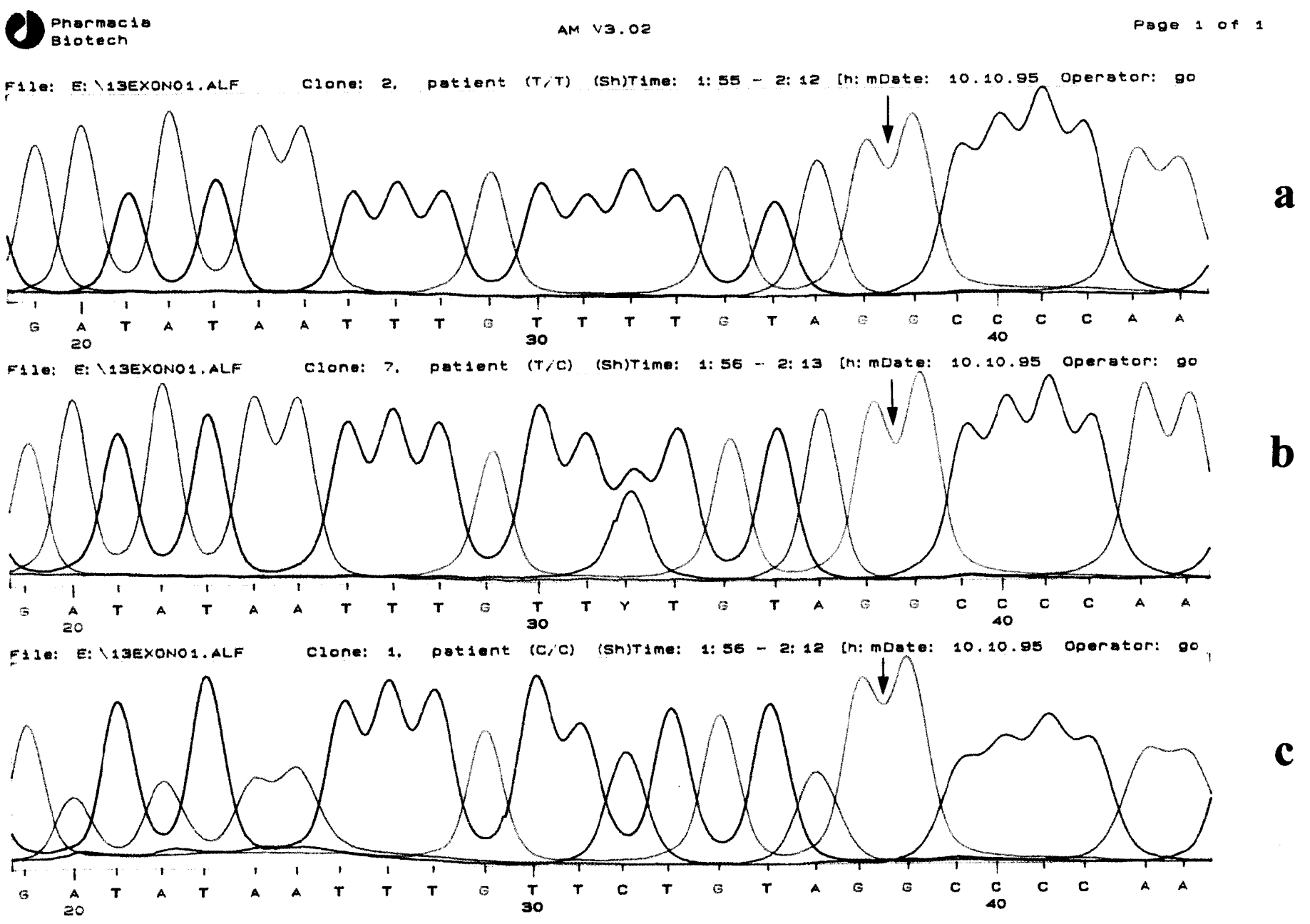

Figure 1. DNA sequence at the -6 splice acceptor site of exon 13 of *hMSH2* in blood lymphocytes from patients with colorectal cancer. (a) A patient with a normal homozygous T/T situation. (b) A patient with a heterozygous T/C situation. (c) A patient with a homozygous C/C situation. The intron 12–exon 13 junction is indicated by an arrow.

Table 1. Prevalence of the intronic germline hMSH2 *T→C transition six bases upstream of exon 13 in patients and controls*

	106 patients with sporadic CRC	125 healthy controls
Normal (T/T)*	77 (73%)	112 (90%)
Heterozygous (T/C)*	28 (26%)	13 (10%)
Homozygous (C/C)*	1 (1%)	0

*Determined in constitutional tissue (blood lymphocytes).

MATERIALS AND METHODS

Patients

A total of 106 consecutive patients from the region of Dresden with histologically proven CRC referred to our hospital for surgery or follow-up were enrolled in the study. After informed consent was obtained, all patients were interviewed with regard to a family history of CRC. A family history of CRC or HNPCC-related tumours (whole gastrointestinal tract, endometrium, pancreas, urinary tract, ovary) was defined as positive if at least one first-degree relative was reported with CRC or HNPCC-related tumours.

No patient fulfilled the Amsterdam criteria for HNPCC [5], and no case of familial adenomatous polyposis coli was detected. Median age at first occurrence of disease in the CRC group was 62 years (range 22–93 years). The control group consisted of 125 anonymous, healthy blood donors with a median age of 44 years (range 18–65 years). Blood donation at the Dresden Red Cross institution is unpaid, which minimises possible selection bias when choosing blood donors as controls [16]. All blood donors were from the same region (Dresden) as the CRC patients; however, although age and sex were known, no family history was available from the blood donors.

DNA sequence analysis

DNA from blood lymphocytes and tumour tissue was isolated using standard methods (QIAamp® blood and tissue kit, Qiagen, Hilden, Germany). Tumour tissue was obtained at surgery and immediately frozen in liquid nitrogen. Exon 13 of *hMSH2* with intron–exon borders was amplified by polymerase chain reaction (PCR) using conditions and primer sequences as described by Kolodner and associates [4]. Single-strand template DNA was isolated using 40 µl PCR product and 20 µl Dynabeads® M-280 (Dynal, Hamburg, Germany). After sequencing with fluorescent-labelled universal primer (AutoRead® Kit, Pharmacia Biotech, Freiburg, Germany), the products were separated by electrophoresis in a 6% polyacrylamide gel and detected by laser fluorescence (A.L.F.®, Pharmacia Biotech, Freiburg, Germany). Positive results (T/C or C/C) were confirmed by repeated testing.

Assessment of MIN

Using fluorescent-labelled primers, four $(CA)_n$ microsatellite loci, Mfd 26 (18q, *DCC* gene) [8], Mfd 27 (5q, *APC* gene) [8], 635/636 (15q, *β-actin* gene) [8] and TP 53(17p, *TP53* gene) [17] were PCR amplified. After that, the amplified microsatellite sequences of constitutional and tumour DNA were separated electrophoretically and detected as described above [18]. Tumour DNA was classified as MIN-positive if it revealed any novel alleles compared with constitutional DNA in at least one out of the four markers tested.

Isolation of hMSH2 *mRNA and cDNA sequencing*

The QuickPrep Micro mRNA Purification Kit® (Pharmacia Biotech, Freiburg, Germany) was used to extract mRNA from blood lymphocytes, tumour tissue and the glioblastoma cell line A172 P5, which was obtained from the Deutsches Krebsforschungszentrum, Heidelberg, Germany. cDNA was generated from poly(A)$^+$mRNA using reverse transcriptase and random hexamers according to manufacturer's instructions (First-Strand cDNA Synthesis Kit®, Pharmacia Biotech, Freiburg, Germany). RT–PCR was performed to amplify *hMSH2* coding sequences from exon 12 (forward1-primer: 5'GGC ATG CTT GTG TTG AAG TTC3') to exon 14 (reverse1-primer: 5'CTG ATA TAG CCC ATG CTA ACC C3'; fragment size: 390 bp) and to the 3' untranslated region (reverse2-primer: 5'GCT TAT CAA TAT TAC CTT CAT TCC3'; 931 bp), respectively. The PCR conditions used were the same as mentioned above. For sequencing of cDNA products, the forward1-primer was applied.

Statistical methods

In this case control study, the χ^2 test was performed with a P value < 0.01 regarded as statistically significant. The odds ratio was calculated to estimate relative risk.

RESULTS

A total of 106 patients with sporadic CRC and 125 healthy controls were analysed for the intronic germline T→C transition six bases upstream of exon 13 of *hMSH2*. Out of the 106 patients, 28 were heterozygous (T/C) and 1 homozygous (C/C) for the T→C transition (27%). In contrast, only 13 out of 125 controls (10%) showed a heterozygous situation ($P < 0.001$; odds ratio 3.2, 95% confidence interval 1.58–6.63) (Figure 1 and Table 1).

Tumour tissue was available from 43 out of the 106 patients with CRC, among them 12 patients heterozygous for

Table 2. Features of patients with and without the intronic hMSH2 *T→C transition six bases upstream of exon 13*

	106 patients with sporadic CRC	
	29 patients with T→C*	77 patients without T→C*
Median age and range (years) at diagnosis	65 [38–84]	61 [22–93]
Right-sided CRC†	6 (21%)	18 (23%)
Family history of CRC†	3 (10%)	6 (8%)
Family history of tumours associated with HNPCC†	7 (24%)	20 (26%)
MIN positive‡	3/12 (25%)	9/31 (29%)

*Determined in constitutional tissue (blood lymphocytes). †For definitions: see Results. ‡Microsatellite instability; for definitions: see Materials and Methods.

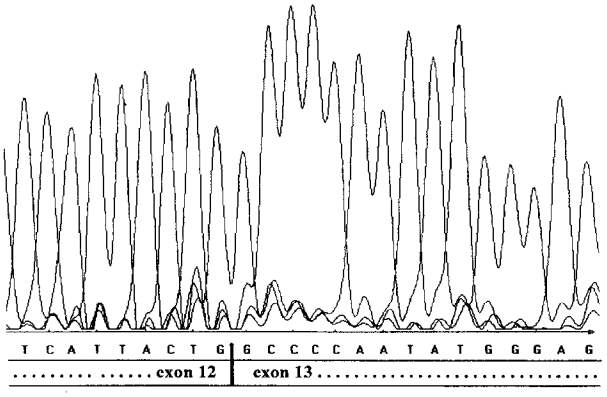

Figure 2. Wild-type cDNA sequence at the exon 12–13 border of *hMSH2* RT–PCR product from the brain tumour cell line A172 P5, which is homo- or hemizygous (C/C or C/-) at the -6 splice acceptor site of exon 13 of *hMSH2*. The same result was obtained with heterozygous (T/C) tumour tissue from 3 patients with CRC and homozygous (C/C) blood lymphocytes from a patient with CRC.

the germline T→C transition. Tumour DNA of the 43 patients was always the same as constitutional DNA at the transition site except for one patient, who appeared to be heterozygous in constitutional DNA and hemizygous (C/-) in tumour DNA.

In patients with CRC, there was no significant age difference between carriers and non-carriers of the transition (Table 2). In 24 patients (23%), the tumour location was right-sided, i.e., proximal to the transverse colon. No significant difference in tumour location was observed between patients with and without the transition (Table 2). A positive family history of CRC in at least one first-degree relative was obtained in 9 (8%) patients. Defining family history as positive by the occurrence of common HNPCC-related tumours (whole gastrointestinal tract, endometrium, pancreas, gall bladder, urinary tract) in at least one first-degree relative, 27 patients (25%) fulfilled these criteria. Independently of the definition used, there was no significant difference between patients with and without the transition (Table 2). Of the 43 tumours available for MIN assessment, 12 (28%) displayed MIN. No significant difference in MIN status was found between carriers and non-carriers of the transition (Table 2).

RT–PCR was performed using heterozygous (T/C) tumour tissue from 3 patients with CRC, homozygous (C/C) blood lymphocytes from 1 patient with CRC and 1 homo- or hemizygous (C/C or C/-) brain tumour cell line. Visualising the amplified cDNA products electrophoretically in an ethidium bromide-stained agarose gel revealed one single band in all samples tested, indicating that no aberrant transcripts were present. Likewise, cDNA sequencing always revealed the wild-type sequence [3] at the exon 12–13 border (Figure 2).

DISCUSSION

Although the intronic germline T→C transition was not uncommon in the control population (10%), there was a significantly enhanced proportion of patients with sporadic CRC bearing it (27%). Comparing normal subjects to a small subgroup of CRC patients, namely, those with a history of ulcerative colitis, Brentnall and associates [13] reported similar data (Table 3). With regard to prevalence of the transition in the general population, our data concur with those of Froggatt and associates [19] and Leach and associates [20], but are in contrast to those of Hall and associates [21] and Borresen and associates [22], who found the transition in approximately a quarter of healthy Caucasian controls (Table 3). In the normal Japanese population [23], the transition was reported to be even more prevalent (Table 3). Thus, the significance of the T→C transition as a genetic risk factor for sporadic CRC may exhibit geographical/racial variations. Although Hall and associates concluded from their data that the T→C transition most likely represents a sequence variation rather than a true mutation, they found that in HNPCC patients with a definite *hMSH2* germline mutation, the phenotype was more severe if the transition was present in the remaining allele [21].

Only 1 out of 12 evaluable heterozygous (T/C) patients had hemizygous (C/-) tumour tissue, apparently due to loss of heterozygosity (LOH) as evidenced by use of microsatellite markers for the *hMSH2* locus (data not shown). A very rare frequency of LOH at the *hMSH2* locus in patients with CRC has been reported by other authors as well [6, 22].

Since our data suggest that the transition is a genetic risk factor for sporadic CRC in the population investigated, possible associated pathogenic effects were sought. Fishel found the transition as a somatic mutation in two sporadic, mismatch repair-defective CRCs from patients with the normal T/T situation in the germline [3]. In our study, none of the 31 patients without the transition in the germline had acquired it in the tumour. Because the transition is located only six bases upstream of the mRNA splice acceptor site of exon 13 of *hMSH2*, it has been speculated that it might possibly interfere with normal *hMSH2* mRNA splicing [3, 13]. In this case skipping of exon 13, which encompasses the most conserved region of *hMSH2* [3], would occur [14]. Most likely this would destroy *hMSH2* protein function. Consequently, tumours of patients with the germline transition should display a typical HNPCC-like phenotype [15]. To test

Table 3. Prevalence of the germline T→C transition (T/C and C/C) at -6 splice acceptor site of exon 13 of hMSH2 *in healthy controls according to the literature*

Author (year)	Individuals	Prevalence T→C	Ref.
Leach and associates (1993)	20	10% (Caucasians)	[20]
Hall and associates (1994)	114	25% (Caucasians)	[21]
Brentnall and associates (1995)	80	9% (Caucasians)	[13]
Borresen and associates (1995)	30–53	24% (Caucasians)	[22]
Froggatt and associates (1996)	16	13% (Caucasians)	[19]
Shirakawa and associates (1996)	24	33% (Japanese)	[23]
Goessl and associates (1997)	125	10% (Caucasians)	this study

this possibility, CRCs from patients with and without the transition were compared. Both clinical and molecular (MIN) HNPCC-associated features were analysed. However, none of these features was found to be correlated with the transition. The overall rate of MIN in sporadic CRC (28%) was higher than reported by most other authors, which may partly be due to the markers used and different definitions of MIN [7, 8, 24].

The possibility of the transition causing alternative splicing of *hMSH2* mRNA was further investigated by analysis of cDNA generated from heterozygous (T/C) tumour tissue of 3 patients with CRC. Tumour tissue was chosen instead of peripheral blood leucocytes because skipping of exon 13 due to alternative splicing does not necessarily reflect a pathological condition in blood lymphocytes [25]. Neither electrophoresis of RT–PCR products nor cDNA sequencing gave any evidence of aberrant splicing in these heterozygous CRCs. However, since abnormally spliced mRNAs may be subject to rapid degradation, they might be undetectable among normal mRNAs in a heterozygous situation [26, 27]. To exclude this possibility, homozygous (C/C) or hemizygous (C/−) material was chosen for investigation of aberrant splicing. Unfortunately, CRC tumour tissue fulfilling these criteria was not available for generation of cDNA. Therefore, cDNA was generated from a hemi- or homozygous (C/- or C/C) brain tumour cell line and from blood lymphocytes of the only patient who was homozygous (C/C) in the germline. Again, in both cases only one single RT–PCR product was found, exclusively showing the wild-type sequence (Figure 2) [3]. This indicates, that—at least in these cells [25]—the T→C transition did not induce aberrant splicing of *hMSH2* mRNA. Brentnall and associates hypothesised that the transition induces aberrant splicing of *hMSH2* mRNA only to a certain extent, dependent on stress factors (e.g., inflammatory processes such as ulcerative colitis) [13]. Thus, aberrant splicing products might be undetectable among the majority of normally spliced mRNA masking them. Theoretically, it is conceivable that such rare events of alternative splicing nevertheless result in mismatch repair deficiency marked enough to leave somatic mutations in target genes unrepaired, thus possibly leading to CRC [28, 29]; if so, then microsatellite sequences, being especially prone to mutations [30], should be affected as well. However, there was no increased rate of MIN in CRC from patients with the germline transition, thus arguing against any direct effect of the transition itself. This conclusion is supported by the fact that both T and C match consensus sequences at the -6 splice acceptor site [31], making their exchange unlikely to interfere with normal mRNA splicing. Thus, the transition may instead be in linkage disequilibrium with an unknown pathogenic locus nearby. A possible candidate might be the *GTPB* gene located in close vicinity to *hMSH2* [32]. Because mutations of *GTPB* result in only slight MIN with preferentially poly(A) repeats affected [32], more extensive studies are planned to investigate an association between the T→C transition and MIN at poly(A) loci in tumours of CRC patients.

Our findings are an example of a genetic risk factor associated with a predisposition to 'sporadic' CRC in a German population. Further studies are necessary to evaluate the usefulness of this marker in other populations. Identifying disease-associated molecular markers and combining them into a genetic risk profile may greatly facilitate the prediction of individual risk and the possibility of concentrating preventive measures on gene carriers.

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