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Variations in mismatch repair genes and colorectal cancer risk and clinical outcome

Veronika Vymetalkova[1,2,*], Barbara Pardini[3], Fabio Rosa[3], Cornelia Di Gaetano[3,4], Jan Novotny[5], Miroslav Levy[6], Tomas Buchler[6,7], Jana Slyskova[1,2], Ludmila Vodickova[1,2], Alessio Naccarati[1,3] and Pavel Vodicka[1,2]

[1]Department of Molecular Biology of Cancer, Institute of Experimental Medicine, Videnska 1083, 14200 Prague, Czech Republic, [2]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University, Katerinska 32, 12800 Prague, Czech Republic, [3]Human Genetics Foundation, Via Nizza 52, 10126 Turin, Italy, [4]Department of Medical Sciences, University of Turin, Via Verdi 8, 10124 Turin, Italy, [5]Department of Oncology, First Faculty of Medicine, Charles University, Katerinska 32, 12800 Prague, Czech Republic, [6]Department of Surgery, First Faculty of Medicine, Charles University, Katerinska 32, 12800 Prague, Czech Republic and [7]Thomayer University Hospital, Videnska 800, 14059 Prague, Czech Republic

*To whom correspondence should be addressed. Tel: +420 2296 2251; Fax: +420 2 41062782; Email: vpolakova@biomed.cas.cz

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DNA mismatch repair (MMR) deficiency is one of the best understood forms of genetic instability in colorectal cancer (CRC). CRC is routinely cured by 5-fluorouracil (5-FU)-based chemotherapy, with a prognostic effect and resistance to such therapy conferred by MMR status. In this study, we aimed to analyse the effect of genetic variants in classical coding regions or in less-explored predicted microRNA (miRNA)-binding sites in the 3′ untranslated region (3′UTR) of MMR genes on the risk of CRC, prognosis and the efficacy of 5-FU therapy. Four single nucleotide polymorphisms (SNPs) in MMR genes were initially tested for susceptibility to CRC in a case–control study (1095 cases and 1469 healthy controls). Subsequently, the same SNPs were analysed for their role in survival on a subset of patients with complete follow-up. Two SNPs in *MLH3* and *MSH6* were associated with clinical outcome. Among cases with colon and sigmoideum cancer, carriers of the CC genotype of rs108621 in the 3′UTR of *MLH3* showed a significantly increased survival compared to those with the CT + TT genotype (log-rank test, $P = 0.05$). Moreover, this polymorphism was also associated with an increased risk of relapse or metastasis in patients with heterozygous genotype (log-rank test, $P = 0.03$). Patients carrying the CC genotype for *MSH6* rs1800935 (D180D) and not undergoing 5-FU-based chemotherapy showed a decreased number of recurrences (log-rank test, $P = 0.03$). No association with CRC risk was observed. We provide the first evidence that variations in potential miRNA target-binding sites in the 3′UTR of MMR genes may contribute to modulate CRC prognosis and predictivity of therapy.

Introduction

Colorectal cancer (CRC) represents a serious health problem in Central Europe and in particular in the Czech Republic, where the incidence for colon cancer ranks the third highest worldwide, and the incidence of rectal cancer is the highest (1,2). Stage I disease usually can be cured by surgery alone, but patients with high-risk Stage II, III or IV disease are recommended for chemotherapy in the adjuvant or palliative setting (3). 5-Fluorouracil (5-FU)-based chemotherapy improves survival in Stage III colon cancer patients and in rectal cancer patients with Stages II and III (4). However, a substantial proportion of patients with CRC do not benefit from 5-FU-based treatment regimens, whereas treatment-related toxicity may be severe and dose limiting (5). 5-FU, an antimetabolite analogous to the pyrimidine uracil, incorporates either into DNA in the form of fluorinated uracil (FdUTP) or it misbalances the synthesis of thymines (dTTPs) by inhibiting thymidylate synthase, leading to erroneous uracil (dUTP) incorporation (6).

Single nucleotide polymorphisms (SNPs) in genes directly involved in the DNA repair mechanisms have attracted a massive and enthusiastic research in order to determine whether different genotypes are associated with CRC risk (7). It is expected that individuals with profound defects in DNA repair will be at greatly increased risk of cancer and other malignancies (8). Although candidate gene analyses have failed to provide convincing evidence of DNA repair SNPs that are directly involved in predisposition to CRC, some variants in genes such as *MUTYH*, *MSH2* and *MLH1* have been proposed to act as low- or moderate-penetrance susceptibility alleles for CRC (9).

DNA repair pathways are almost all presumably involved in the cell response to CRC treatment. To date, the pathways that appear most relevant in determining the 5-FU outcome are base excision repair (BER) and mismatch repair (MMR). However, mechanistic details remain unexplained, and other pathways have not been exhaustively interrogated (10). MMR is responsible for correcting replication errors such as base:base mismatches and polymerase slippage products (i.e. insertion/deletion loops) at nucleotide repeat sequences (11). MMR also plays an important role in apoptotic signalling in response to DNA damage (12). Specifically, MMR recognition of damaged DNA can signal to the cell death machinery to trigger apoptosis, so that loss of MMR by genetic or epigenetic means can promote a ‘tolerance’ to DNA damage and resistance to chemotherapeutic DNA damaging agents. A number of studies have reported that cells deficient in MMR components, particularly MSH2 and MLH1, are resistant to 5-FU (13–15).

There has been growing evidence that deficient MMR [dMMR; microsatellite instability (MSI)-positive] status is a strong prognostic biomarker for improved outcomes in CRC. Recently, O’Leary and Gilbert (16) considered assessing MMR status as a routine in Stage II CRC. Several meta-analyses have reported an increased overall survival (OS) in dMMR patients (17–19). A retrospective analysis of 1913 patients showed prognostic effect of dMMR in Stage II CRC, highlighting the association with clinical features (19). Although dMMR status may confer strong evidence of a prognostic effect and resistance to 5-FU, it is not yet thoroughly investigated whether genetic variants in genes of this pathway may modulate this response. In this respect, only few studies have considered the importance of SNPs for cancer therapy efficacy and patients survival. Koessler *et al.* (20) observed that SNPs in *MSH2* (rs4638843), *MSH3* (rs33015) and *MSH6* (rs3136245) genes were associated with

worse OS, while rs27385 in *MSH2* was associated with longer survival in CRC patients. Similarly, in pancreatic cancer patients with variant allele in *MSH2* (G322D) and *MSH6* (G39E), polymorphisms were associated with shorter survival (21).

Three SNPs located in the coding region of MMR genes and with amino acid characteristic [rs1799977:A>G (*MLH1* I219V), rs1800932:A>G (*MSH6* P92P) and rs1800935:T>C (*MSH6* D180D)] within CRC susceptibility loci (22) were reanalysed in a recent meta-analysis, which included the present CRC case–control population from the Czech Republic (23). Nonetheless, those SNPs were not investigated on patient´s survival and therapy efficacy.

Recently, we have investigated the role of variations in microRNA (miRNA) target-binding sites in the 3′ untranslated region (3′UTR) of DNA repair genes in terms of association both with CRC risk and survival. Interestingly, SNPs in nucleotide excision repair (24) and BER (25) genes were, respectively, associated with risk and survival. The presence of SNPs within 3′UTRs of target MMR genes could alter the binding with specific miRNAs, modulating gene expression and ultimately affecting cancer susceptibility, prognosis and therapy outcomes (26).

Based on the above considerations, the aim of this study was to investigate the role of polymorphisms both in coding regions and in potential miRNA-binding sites within 3′UTR of MMR genes in modulating the risk of CRC, its progression and prognosis. Understanding the involvement of MMR system in the response of cancer cells to antineoplastic drugs is expected to be very important for the design of personalised therapy regimes and the prediction of therapeutic response in CRC.

Materials and methods

Study population

Blood samples were collected among patients with histologically confirmed CRC, recruited between September 2003 and May 2012 from several oncological departments in the Czech Republic. This study included 1095 CRC patients and 1469 controls that provided biological samples and could be genotyped appropriately. Cases and controls were previously described in details in Refs (24,25).

All subjects were informed and provided written consent to participate in the study and to use their biological samples for genetic analyses, according to the Helsinki declaration. The design of the study was approved by the local ethics committee. Biological, lifestyle and demographic characteristics and potential risk factors for CRC, such as body mass index (BMI), diabetes and family/personal history of cancer, were collected in structured questionnaires.

Survival study on CRC patients

For a subgroup of 866 CRC cases, detailed information were available on clinical data at the time of diagnosis, including location of the tumour, UICC (International Union Against Cancer) tumour–node–metastasis (TNM) system and grade and adjuvant chemotherapy treatment. Information about distant metastasis, relapse and date of death was also collected, with a follow-up until August 31, 2011. However, for 223 CRC cases, no clear or complete information was available; therefore, they were excluded from the analysis. Of the remaining 643 cases, 319 patients were administered with a 5-FU-based adjuvant regimen as first-line postoperative therapy. The therapy consisted of either a Mayo regimen, delivered as a bolus infusion of 5-FU (425 mg/m^2) and leucovorin (10 mg/m^2) for 5 days every 4 weeks six times, or a simplified DeGramond regimen, which consisted of a 2-h intravenous (i.v.) infusion of leucovorin (200 mg/m^2), then a 5-FU i.v. bolus (400 mg/m^2) followed by a 46-h 5-FU continuous i.v. infusion (2400–3000 mg/m^2). Three hundred and twenty-four subjects did not receive any adjuvant chemotherapy after surgery. In this study, the outcome variables measured were 5-FU-based chemotherapy, OS (time from diagnosis till death or censorship) and event-free survival (EFS, time of surgery or end of chemotherapy till date of relapse, death or censorship).

Selection of candidate genes

SNPs in miRNA target-binding sites. From the complete list of all DNA repair genes divided by pathway (available online at http://sciencepark.mdanderson.org/labs/wood/dna_repair_genes.html), 10 MMR genes were identified (last update March 2013). For each gene, SNPs in target-binding sites for miRNAs were investigated by using freely available software MicroSNiPer (http://epicenter.ie-freiburg.mpg.de/services/microsniper/) (27), which interrogates the 3′UTR and predicts if a SNP within the target site will disrupt/eliminate or enhance/create a miRNA-binding site (25). All 16 detected SNPs were tested for minor allele frequency (MAF, >5% in Caucasian populations) in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) in order to reach an appropriate statistical power. The selection was primarily done on the basis of HAPMAP CEU population. Whenever this was not possible, other populations were checked (i.e. 1000 genomes: Phase 1, CEU population). SNPs with the required MAF were further tested for the possibility to be in *linkage disequilibrium* using HaploView (v. 4.2) with the data from HapMap v. 3 (release R2) in the CEU population.

After this selection, three SNPs (rs394592 in *MSH3*, rs108621 in *MLH3* and rs17147225 in *PMS2L3*) in the 3′UTRs of 10 MMR genes were found and fulfilled the required selection criteria.

Additional MMR gene SNPs for survival analysis. We also included in the study three SNPs in the coding region of two MMR genes (rs1799977 in *MLH1* and rs1800932 and rs1800935 in *MSH6*) previously investigated for CRC risk (22,28). Those SNPs have been also tested in our previous study conducted on the present CRC case–control population from the Czech Republic, but no association for CRC risk emerged (23). Hereby, we have tested those SNPs only for the survival analysis.

SNP genotyping

The genomic DNA was isolated from peripheral blood lymphocytes using standard procedures. The DNA samples from cases and controls were randomly placed on plates where an equal number of cases and controls could be run simultaneously. Genotyping of the selected SNPs was carried out by using the KASPar chemistry of LGC Genomics (Hoddesdon, Herts, UK: http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/), as previously described in Landi *et al.* (29). For quality control purposes, duplicate samples (5% of the total numbers of samples) were repeated for each SNP and no template controls were included in each plate.

Statistical analyses

Chi-square test (1 degree of freedom), with a type I error threshold set at $\alpha = 0.05$, was used to verify whether the genotypes were in Hardy–Weinberg equilibrium in controls. The multivariate logistic regression analysis was used to test the association between genotypes and risk of CRC. The covariates analysed for inclusion in the multivariate model were sex, age, smoking habit (non-smokers vs. smokers and ex-smokers), BMI, any positive familial history of CRC, education level (high, intermediate and low) and living area (country, town neighbourhood and town). The association between SNPs and CRC risk was calculated by estimating the odds ratios (ORs) and their 95% confidence intervals (CIs), adjusted for both continuous and discrete covariates. For all the genotypes, regression coefficients for the additive model were estimated. For all SNPs with significant *P* values per genotype, the best model (dominant or recessive) was calculated. Statistical analyses were performed using R (http://www.rproject.org).

OS in CRC patients was evaluated using the date of death or the date of the end of the study (August 31, 2011) as the end point of follow-up. For the EFS, in patients who did not have distant metastasis at the time of diagnosis, date of relapse, death or end of the study was used as the end point of follow-up. EFS was defined as the time from surgery/end of therapy to the occurrence of distant metastasis, recurrence or death, whichever came first. The relative risk of death and recurrence was estimated as hazard ratio (HR) using Cox regression. The survival curves for overall and EFS were derived by the Kaplan–Meier method. Multivariate survival analyses were adjusted for age, gender, T, N, M and chemotherapy. Statistical analyses were performed using R (R version 2.14-2, Survival package).

Results

SNP selection

Sixteen SNPs were identified within the 3′UTRs of seven genes (*MLH3*, *MSH3*, *MSH4*, *MSH6*, *PMS1*, *PMS2* and *PMS2L3*) out of 10 genes involved in the MMR pathway; the remaining three genes (*MLH1*, *MSH2* and *MSH5*) did not present any SNP.

After testing for MAF in Caucasian populations, nine SNPs resulting less frequent were excluded from the selection.

Additionally, five SNPs were found to be in the same haplotype block (D′ 1, r^2 1) of one identified SNP (*MLH3* rs108621 with rs108622, rs175049, rs398896 and rs424120); therefore, in our study, we considered only rs108621.

Finally, three SNPs (rs394592 in *MSH3*, rs108621 in *MLH3* and rs17147225 in *PMS2L3*) passed the selection and were analysed in the study. After genotyping step, the assay for SNP rs394592 failed to pass its validation. The data from this assay showed only non-specific amplification and so could not be used to generate any genotyping results. On the other side, the other two SNPs were genotyped successfully, but rs17147225 resulted monomorphic (only GG genotype in all subjects) in our population.

Case–control study

Genotyping analysis was performed on 1095 CRC patients, among whom approximately two-thirds were diagnosed with a tumour in colon and sigmoideum (389 and 336, respectively), while the rest with rectal cancer (370). Out of the 1469 controls, 688 were cancer-free colonoscopy inspected controls (Control group 1) and 781 were healthy blood donor volunteers (Control group 2). Compared to subjects of both control groups, CRC cases were more likely to be older, have a slightly higher BMI while, compared to the Control group 2, they were more likely to have a positive family history of CRC and lower formal education (Table I).

The genotype screening was performed simultaneously for cases and controls. The results were regularly confirmed by random re-genotyping of >5% of the samples for each polymorphism, which yielded concordant results. The genotypes with unclear results were excluded from the data. The distribution of genotypes within the selected genes in the controls was in agreement with Hardy–Weinberg equilibrium.

In this study, rs108621 was not associated with CRC risk. To overcome confounding effect of age (mean age in cases = 61.7 ± 10.8 years, mean age in controls = 50.6 ± 12.2 years, *t*-test = 24.4, $P \leq 0.0001$), cases and controls were matched by age quartiles through bootstrap sampling (10 repetitions). For each subset, the whole association analysis was repeated, and the results averaged. With this approach, we could ascertain that no changes were observed in the 10 different re-samplings. Additionally, we repeated the analysis on a subgroup of 800 cases and 800 controls matched for sex and age (±5 years), and we obtained similar results as in the whole group of study (after matching, mean age in cases was 59.0 years and mean age in controls was 57.7 years).

Results for the other three SNPs in MMR genes (rs1799977 in *MLH1* and rs1800932 and rs1800935 in *MSH6*) were previously published, and no association with the risk of CRC was found for any of the studied polymorphisms in our study population (23).

Survival analysis

The average (median) OS and EFS for the studied population were 87.8 (72.0) and 83.0 (68.1) months, respectively. In the preliminary univariate assessment of covariates, several parameters, such as gender, age, BMI, smoking habit, T, N, M status and chemotherapy treatment, were found to be associated with OS (Table II). In particular, advanced age, male gender and current smoking status resulted in association

Table I. Characteristics of the study population

	CRC cases	Control group I	Control group II	All controls	OR (95% CI)	*P* value
All subjects	1095	688	781	1469		
Diagnosis						
Colon cancer	725	–	–	–		
Rectal cancer	370	–	–	–		
Age (years)						
≤47	94	164	427	591	Reference	
48–55	208	145	277	422	3.10 (2.36–4.09)	**<0.01**
56–65	370	209	77	286	8.13 (6.25–10.66)	**<0.01**
>65	423	173	0	173	15.37 (11.66–20.44)	**<0.01**
Sex						
Females	435	317	343	660	Reference	
Males	660	371	438	809	1.23 (1.05–1.45)	**0.01**
BMI						
≤23.7	184	154	215	369	Reference	
23.7–26.2	192	147	213	360	1.07 (0.83–1.37)	0.61
26.3–28.9	226	139	184	323	1.40 (1.10–1.79)	**0.01**
>28.9	222	172	157	329	1.35 (1.06–1.73)	**0.02**
Smoking habit						
No	536	364	451	815	Reference	
Yes[a]	501	254	327	581	1.31 (1.12–1.54)	**<0.01**
Family history of CRC						
No	726	486	718	1204	Reference	
Yes	144	90	52	142	1.68 (1.31–2.16)	**<0.01**
Living area						
City	511	338	614	952	Reference	
Suburbs	128	118	53	171	1.39 (1.08–1.79)	**0.01**
Countryside	242	157	112	270	1.67 (1.36–2.05)	**<0.01**
Education						
Basic	266	171	53	224	Reference	
Medium	469	327	492	820	0.48 (0.39–0.59)	**<0.01**
High	138	114	231	345	0.34 (0.26–0.44)	**<0.01**

Significant results are given in bold. Numbers may not add up to 100% of available subjects because of missing data.
[a]Ex-smokers are included into this group.

Table II. Clinical and anamnestic characteristics significantly affecting OS and EFS of the CRC patients with complete follow-up (Cox regression)

	Patients[a]	OS		EFS	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex					
Females	357	Reference		Reference	
Males	509	1.54 (1.22–1.94)	**<0.01**	1.43 (1.14–1.80)	**<0.01**
Age (years)					
≤55	235	Reference		Reference	
56–62	203	1.40 (1.00–1.94)	**0.05**	1.32 (0.97–1.78)	0.07
63–69	221	1.33 (0.96–1.84)	0.08	1.07 (0.79–1.46)	0.66
>69	207	1.87 (1.37–2.57)	**<0.01**	1.05 (0.76–1.45)	0.77
BMI					
≤24	162	Reference		Reference	
24.1–26.5	157	0.86 (0.62–1.21)	0.39	0.93 (0.67–1.28)	0.66
26.6–29.1	163	0.68 (0.48–0.96)	**0.03**	0.63 (0.44–0.89)	**0.01**
>29.1	155	0.61 (0.43–0.87)	**0.01**	0.77 (0.55–1.08)	0.13
Smoking habit					
No	428	Reference		Reference	
Yes[b]	388	1.16 (0.93–1.45)	0.19	1.20 (0.96–1.50)	0.10
Yes[c]	116	1.54 (1.13–2.09)	**0.01**	1.26 (0.91–1.75)	0.16
Family history of CRC					
No	545	Reference		Reference	
Yes	111	1.07 (0.77–1.49)	0.67	1.09 (0.79–1.49)	0.60
pT					
1	32	Reference		Reference	
2	130	2.66 (0.82–8.67)	0.10	2.18 (0.77–6.16)	0.14
3	420	5.84 (1.87–18.28)	**<0.01**	5.26 (1.95–14.16)	**<0.01**
4	90	12.30 (3.85–39.23)	**<0.01**	8.09 (2.93–22.33)	**<0.01**
pN					
0	359	Reference		Reference	
1	213	2.04 (1.56–2.67)	**<0.01**	1.76 (1.35–2.30)	**<0.01**
2	51	4.69 (3.17–6.94)	**<0.01**	3.51 (2.42–5.07)	**<0.01**
pM					
0	545	Reference		Reference	
1	150	4.67 (3.68–5.93)	**<0.01**	4.11 (3.28–5.15)	**<0.01**
5-FU-based chemotherapy					
Yes	319	Reference			
No	324	1.66 (1.29–2.12)	**<0.01**	1.14 (0.90–1.45)	0.27

Significant results are given in bold. pT, pN, pM—pathological assesment of primary tumor (T), regional lymph nodes (N), distant metastasis (M).
[a]Numbers may not add up to 100% of available subjects because of missing information.
[b]Ex-smokers are included into this group.
[c]Ex-smokers excluded.

with a shortened OS. Men showed also a higher risk of relapse or metastasis (OS—HR: 1.54; 95% CI: 1.22–1.94; $P < 0.01$; EFS—HR: 1.43; 95% CI: 1.14–1.80; $P < 0.01$). Conversely, higher BMI was related with both an increased survival and a lower risk to develop relapse or metastasis. Four established prognostic factors (T, N, M status and chemotherapy treatment) were associated with patient survival. Moreover, TNM status was also associated with an increased risk of recurrence.

Overall, no association with survival was observed for all analysed SNPs, adjusting for significant covariates (Supplementary Table I, available at *Mutagenesis* Online). After stratification of patients according to tumour location, the carriers of the homozygous variant CC genotype of rs108621 in *MLH3* affected by malignancy in colon and sigmoideum showed a longer survival in a recessive model (log-rank test for the recessive model, $P = 0.05$) (data not shown).

A lower risk of relapse or metastasis was observed for patients carrying the CT genotype of *MLH3* rs108621, both considering the whole group of cases or only subjects with malignancy in colon and sigmoideum (log-rank test for the co-dominant model for CT genotype, $P = 0.03$ and 0.04, respectively) (Figure 1A and B). After stratification for 5-FU-based chemotherapy, EFS was associated with rs1800935 in *MSH6* gene (log-rank test, $P = 0.03$). In particular, we have observed a worse survival for those patients carrying the CC genotype and not undergoing 5-FU-based chemotherapy (Figure 2). No association was observed for rs1799977 in *MLH1* gene and rs1800932 in *MSH6* gene.

Further stratification of patients according to stage of disease did not show any significant associations with the OS or EFS (data not shown).

Discussion

In this study, we have investigated whether genetic variants in MMR genes were associated with CRC susceptibility and prognosis and may modulate the response to chemotherapeutics. We aimed to explore this assumption at two different levels: variation in classical coding regions of genes and less explored variation in 3′UTR in predicted miRNA-binding sites. The main and novel finding of this study is that SNPs in two MMR genes, *MLH3* and *MSH6*, were associated with patients' survival. In particular, among cases with colon and sigmoideum cancer, carriers of the CC genotype of rs108621 in *MLH3* gene showed a significantly increased OS compared to the other genotypes. Surprisingly, patients with heterozygous

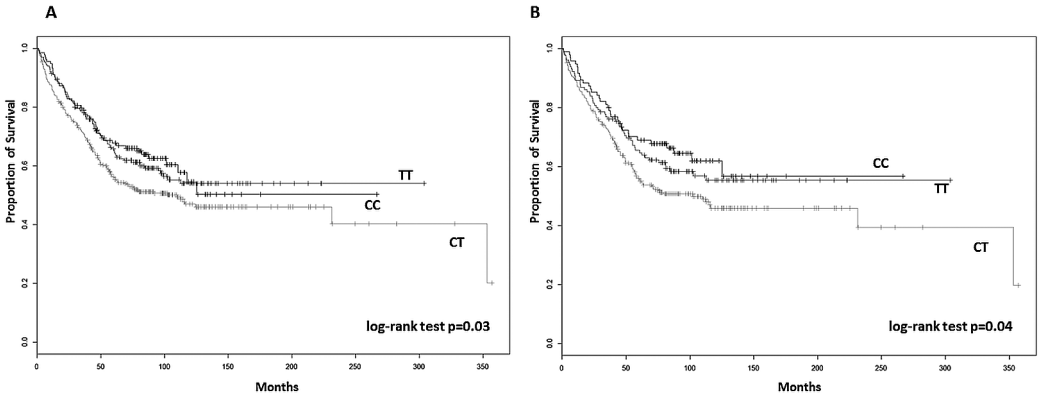

Fig. 1. Kaplan–Meier EFS curves stratified for *MLH3* rs108621 in all CRC patients (**A**) and in colon and sigmoideum cancer patients (**B**).

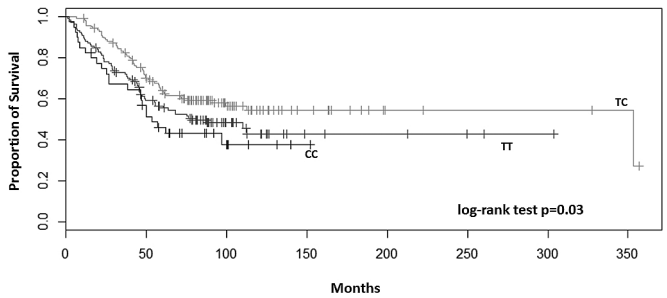

Fig. 2. Kaplan–Meier EFS curves of CRC patients stratified for *MSH6* rs1800935 and not undergoing 5-FU-based chemotherapy.

genotype for this SNP were also associated with an increased risk of relapse or metastasis. Additionally, an increased risk of relapse or metastasis was observed also for those patients carrying the CC genotype for rs1800935 in *MSH6* and not undergoing 5-FU-based chemotherapy. These results, though very promising, need further investigations in a larger study group of patients.

MLH3 is a member of the MutL-homolog (MLH) family of DNA MMR genes. Mammalian *MLH3* participates in tumour suppression, but the precise mechanisms have not been yet fully addressed. Based on the literature search, Koessler *et al.* (20) found no evidence for association of other common variants of *MLH3* with survival after diagnosis of CRC. On the other hand, Chen *et al.* (30) observed in mice that *MLH3* deficiency alone causes microsatellite instability, impaired DNA-damage response, increased tumour susceptibility in gastrointestinal and extra-gastrointestinal tissues and early mortality. *MSH6* is part of MutS heterodimer, which initiates MMR by binding to a mismatch and then forming a complex with MutL alpha heterodimer. The SNP rs1800935 in *MSH6* analysed in our study was also previously associated with worse OS in patients with locally advanced or metastatic pancreatic cancer (21).

Since regulation of and coordination among genes involved in the various DNA repair pathways are fundamental for maintaining genome stability, post-transcriptional gene regulation by miRNAs could be critical in these processes (31). An increasing body of evidence indicates the possibility to study altered miRNA expression as diagnostic, prognostic and predictive clinical marker (32,33). In this respect, the presence of SNPs in 3′UTRs of MMR genes located at miRNA target sites can either modulate the binding or create novel binding sites exerting their influence in modulating gene expression and ultimately affecting cancer susceptibility (24). Less is known about the effect of variations in target sites of DNA repair genes on survival and the effect of therapy via miRNAs modulation (34). Recently, SNPs in 3′UTRs of BER genes *SMUG1* and *NEIL2* were found associated with OS in CRC patients (25). SNPs in predicted miRNA-binding sites of MMR genes, as we observed in *MLH3* gene, may be important factors for modulating CRC prognosis. It should be noticed that rs108621 in *MLH3* is in linkage disequilibrium with four other SNPs (rs108622, rs175049, rs398896 and rs424120). Interestingly, both alleles of rs108621 represent a predicted target for binding of nine different miRNAs, in contrast to the other SNPs that show a considerably lower number of binding miRNAs. However, the presence of high density of SNPs could also contribute to a different combination of miRNAs interacting in the particular region. This fact may additionally affect the modulation of post-transcriptional regulation mediated by individual SNPs. Therefore, at present, we cannot exclude that the observed clinical phenotypes may be the result of different combinations of miRNAs binding to one of such predicted SNP. Moreover, very recent report suggests that variations in gene regions other than 3′UTRs may also affect binding of miRNAs (35).

Concerning the 5-FU treatment, it is still unclear to what extent MMR genes contribute to 5-FU cellular sensitivity. Several studies have found that patients with MMR proficient tumours (i.e. showing no MSI) more significantly benefit from 5-FU treatment (36–38), while other studies report no obvious difference in 5-FU response and MSI status (17,39). As studies are ambiguous regarding the clinical efficacy of 5-FU treatment as predicted by MMR genotype, it stands to reason that other factors play at minimum equally vital roles in determining individual responsiveness to 5-FU exposure (10). Regrettably, we missed information on MSI status for a large part of our cancer population, so a further stratification in our study for MSI

was not possible. Although a prognostic relevance and a resistance to 5-FU due to dMMR status (MSI-positive) in Stage II CRC patients were reported in other studies (19,40), we did not observe a stage-related association for the gene variants analysed in this study.

We are aware of certain limitations of this study, such as differences among cases and controls for some parameters (i.e. mean age and BMI). The inclusion of 'colonoscopically negative' individuals ensured disease-free control individuals because a negative colonoscopy result is the best available proof of the CRC absence (41). On the other hand, since this group of individuals may not necessarily represent the general population, we also included healthy individuals who were overall younger from among volunteers recruited from blood centres. We attempted to control the putative age effect by matching cases and controls by age quartiles through bootstrap sampling and no changes were observed in the 10 different re-samplings.

We also did not apply any correction for multiple testing analyses. With four SNPs, applying Bonferroni's correction (i.e. adjusted threshold of significance $P < 0.0125$), a majority of the observed associations would be lost. On the other hand, such conservative correction may not be required, considering the exploratory nature of our study and the fact that all the SNPs were selected for their high prior probability of functional significance based on differential binding of miRNAs to their predicted polymorphic target sites.

Moreover, our study population was adequate to investigate associations with a sufficient power. In fact, considering a fixed power of the study = 0.8, with the available number of subjects and the observed MAF for the investigated SNPs, the minimum detectable OR (for the case–control study) and HR (for the survival study) to detect significant differences were 1.13–1.21 and 1.28–1.45, respectively. Our results encourage further investigations in larger set of samples with complete information on follow-up on the role of polymorphisms within miRNA-binding sites and miRNA-dependent gene regulation as a possible functional significance of variation in humans.

To our knowledge, this is the first study investigating in a large group of patients the role of SNPs residing in miRNA target sites of MMR genes in association with CRC survival and with the 5-FU-based chemotherapy. Moreover, patients were collected from the same centres (with follow-up data collected by the same physicists) and were highly homogeneous for their ancestry (all from Czech Republic), thus with the exclusion of possible population stratifications.

In conclusion, we identified plausible candidate SNPs in MMR genes that are associated with clinical outcome in patients with CRC and survival. Additional studies are warranted to establish and validate a genetic risk prediction model for CRC. Such studies could identify some genes as potential therapeutic targets in the treatment of this frequent disease.

Supplementary data

Supplementary Table I is available at *Mutagenesis* Online.

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