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ORIGINAL ARTICLE

Microsatellite instability and promoter hypermethylation of *MLH1* and *MSH2* in patients with sporadic colorectal cancer

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Summary

Purpose: *Inactivation of the genes involved in DNA mismatch repair (MMR) is associated with microsatellite instability (MSI) and loss of heterozygosity (LOH). The aim of the current study was to assess the presence of MSI and promoter hypermethylation of MLH1 and MSH2 in Bulgarian patients with sporadic colorectal cancer (CRC) and to analyze their possible effect on the development, progression and prognosis of the disease.*

Methods: *We examined MSI in 126 patients with sporadic CRC and the methylation status of the MLH1 and MSH2 promoter regions in the cases with MSI/LOH by using a panel of 5 microsatellite markers (BAT26, D5S346, D18S35, D2S123 and FGA) and methyl-specific PCR (MSP) of bisulfite converted DNA.*

Results: *MSI/LOH was found in 36 (28.6%) patients. Among them, 30 were analyzed for promoter hypermethylation of MLH1 and we detected hypermethylation in 15 (50%) of them, whereas promoter hypermethylation of MSH2 was observed only in one case. The presence of MSI/LOH was associated with younger age (p=0.002), more advanced stage (III/IV stage) (p=0.029), lower degree of differentiation (p=0.001), and right-sided tumor localization (p=0.0002), but not with overall survival (log rank, p=0.566).*

Conclusion: *Our data suggest that sporadic CRCs with MSI/LOH are more aggressive, develop earlier and progress faster to more advanced stage. The most frequent cause of failure of DNA MMR system appeared to be the hypermethylation of CpG islands of the promoter region of MLH1, whereas the methylation of MSH2 was a rare event.*

Key words: colorectal cancer, hypermethylation, MLH1, MSH2, microsatellite instability, loss of heterozygosity, prognosis

Introduction

CRC in Bulgaria is the second most frequent malignant disease after the breast cancer for women and lung cancer for men, with more than 4000 new cases and 2443 deaths in 2006 [1]. Due to its social significance, the factors involved in the colorectal carcinogenesis, progression and prognosis have been the focus of extensive research studies for a long time.

CRC arises through the cumulative effects of inherited genetic susceptibility and environmental exposures. These two sets of factors interact and cause CRC by either inducing or enabling the progressive accumulation of gene mutations and epigenetic alterations [2]. Various genetic alterations are involved in the carcinogenesis of CRC and substantiate the adenoma-carcinoma sequence theory [3,4]. The most common pathway for developing CRC, occurring in about 85% of the sporadic cases, involves accumulation of multiple gene mutations and it is referred to as chromosome instability pathway (CIN) or microsatellite stable pathway (MSS) [2]. Both activation of oncogenes such as *K-ras*, and inactivation of tumor suppressor genes as *p53, APC, DCC* have been identified and approximately 50% of CRC

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can be explained by these mechanisms [5-7]. In addition, approximately 15% of sporadic CRC show DNA MSI, which is a result from the failure of DNA MMR system [2,8,9].

MSI, formerly known as replication error phenotype (RER), is the hallmark of MMR deficiency. MMR deficiency in hereditary and sporadic colorectal cancers occurs mainly through two different mechanisms. Germ-line mutations in some of the mismatch repair enzyme genes (mainly *MLH1*, *MSH2* and *MSH6*) are usually the cause of hereditary colorectal cancers, such as hereditary non-polyposis colorectal cancer (HNPCC) [8-10]. As a consequence of such mutations the lifetime risk of development of CRC is more than 80%, compared to 5-6% in the general population [11].

In sporadic CRC the MSI is due to the loss of expression of a mismatch repair gene (most commonly *MLH1*) because of epigenetic silencing. The epigenetic silencing occurs mainly by aberrant methylation (hypermethylation) of CpG dinucleotides in the promoter regions of genes which prevents gene transcription causing deficiency in protein expression [12,13]. However, other mechanisms associated with MSI have been proposed as well, and include somatic mutations in MMR enzyme genes (*MLH1, MSH2, MSH6*) [14], alteration in histone modification [15] and aberrant expression of microRNAs (miRNAs) [16].

An increasing number of reports describe that sporadic CRC with MSI differs from sporadic MSS cancers in molecular, pathological and clinical aspects [11,17,18]. Conflicting data exist, however, concerning the impact of MSI on the progression, prognosis and therapeutic response to adjuvant chemotherapy of patients with CRC [11,19-22].

The goal of the current study was to assess the presence of MSI and promoter hypermethylation of *MLH1* and *MSH2* in patients with sporadic colorectal cancer and to analyze their possible effect on the development, progression and prognosis of the disease.

Methods

Patients

In the current study enrolled were 165 patients with primary CRC: 110 (66.7%) suffered from colonic and 55 (33.3%) from rectal cancers. Part of the patients (n=114) involved in the analysis had undergone tumor resection between January 2001 and December 2005 at the University Hospital, Medical Faculty, Trakia University, Stara Zagora, whereas the remaining 51 patients were operated between 1998 and 2002 at the Department of Gastroenterology, University Hospital "Queen Giovana", Medical University, Sofia, Bulgaria.

The patient population consisted of 102 (61.8%) males and 63 (38.2%) females, aged between 36 to 95 years (median 65.5). The available clinical data of the patients are presented in Table 1.

Clinical data for survival analyses obtained from the archival records were available for 94 patients. All of them were followed-up until the 1st of March, 2009. The median follow-up period for the whole patient population was 37.1 months after surgery (range 0.2 - 137.6). At the end of follow-up, 45 (47.9%) of the patients were alive, with a median survival period of 76.9 months (range 26.8 - 137.6). Forty-nine patients (52.1%) died with a median survival period of 16.0 months (range 0.2 - 105.2).

The study was carried out according to the requirements of the University Hospital Ethics Committees of Trakia University and Medical University, Sofia, and written informed consent was obtained from the patients.

Biological materials

Assessment of MSI requires genomic DNA from tumor tissue and control genomic DNA from normal tissue or blood cells. In this respect, genomic DNA from 41 patients was obtained from routinely processed for-

Table 1. Main clinical parameters available of the patients with colorectal cancer

Parameters	*Patients, n (%)*
Gender (n=137)	
Male	84 (61.3)
Female	53 (38.7)
Age (years; n=137)	
Mean	64.2±11
Median (range)	65.8 (36-95)
Location of the primary tumor (n=137)	
Colon	91 (66.4)
Rectum	46 (33.6)
TNM stage (n=83)	
I	5 (6.0)
II	38 (45.8)
III	17 (20.5)
IV	23 (27.7)
Presence of metastases in regional lymph nodes (n=79)	
No	53 (67.1)
Yes	26 (32.9)
ECOG performance status (n=72)	
2	63 (87.5)
3	2 (2.8)
4	7 (9.7)
Chemotherapy (n=69)	
Yes	41 (59.4)
No	28 (40.6)

malin-fixed paraffin-embedded biopsy pairs containing tumor and normal tissue, respectively. For 52 of the patients, fresh frozen paired biopsies of normal mucosa and tumor tissue were available and for the remaining 72 patients there were paraffin-embedded biopsies containing only tumor tissue. From 51 of those latter patients, peripheral venous blood as a control unaffected tissue was obtained. Thus, for 21 of the patients we isolated genomic DNA only from tumor tissue, and those patients were excluded from the MSI analyses.

DNA isolation

Genomic DNA from routinely processed formalin-fixed paraffin-embedded biopsies was extracted after dewaxing and rehydration of 4-6 10 μm-thick slides by using two different methods [23]: (i) the standard method, implicating the proteinase K digestion followed by phenol-chloroform-isoamyle alcohol extraction and absolute ethanol precipitation of DNA; and (ii) a DNA extraction kit (Macheray-Nagel, Germany), based on the proteinase K digestion followed by silica column filtration and collection of DNA. Genomic DNA from fresh frozen biopsies was also isolated using the DNA extraction kit (Macheray-Nagel, Germany).

DNA from whole venous blood was isolated applying the conventional proteinase K digestion followed by protein precipitation with oversaturated solution of NaCl, and deposit of genomic DNA with absolute ethanol [24]. In some cases a commercial kit for isolation of genomic DNA from blood (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma, USA) was also used.

MSI analysis

A panel of 5 polymorphic markers, BAT26, D2S123, D5S346, D18S35, and FGA, were analyzed for MSI, as described before [24,25]. The microsatellite markers included one mono-nucleotide (poly-A in BAT26), 3 di-nucleotide (poly-CA in D5S346, poly-AC in D2S123, and poly-CA in D18S35), and one tetra-nucleotide repeat markers (FGA). The microsatellite markers were amplified from both normal and tumor DNA samples and electrophoresis was performed on an automated fluorescence sequencer (ABI 3130xl, Applied Biosystems), CA, USA. MSI was defined by the appearance of different alleles in the tumor DNA when compared to the corresponding normal DNA (Figures 1 and 2). When the area under the peak of an allele was significantly reduced (more than 50%) in PCR with tumor DNA compared to those with control DNA, the change was defined as LOH (Figure 3). Tumors that displayed microsatellite alterations at 2 or more loci were classified as MSI-H (MSI high), those showing difference in one locus were defined as MSI-L (MSI low), and all the rest as MSI stable (MSS).

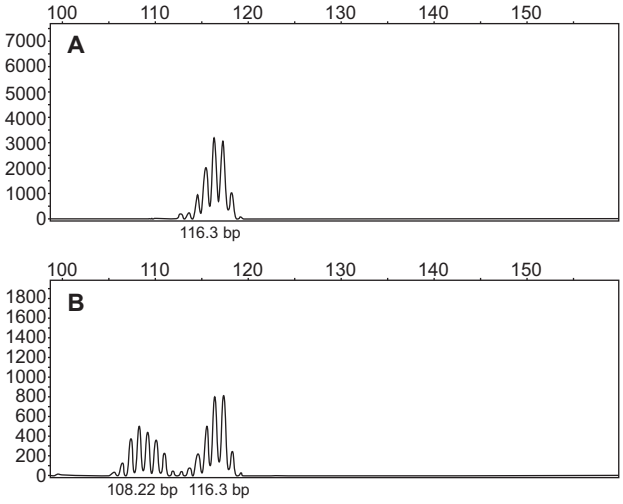

Figure 1. MSI of BAT26 marker in a patient with CRC, demonstrated by deletion of 8bp: there are two PCR products from tumor tissue with a length of 108.22 bp and 116.3 bp, respectively **(B)**, whereas there is only one PCR product from paired normal tissue with a length of 116.3 bp **(A)**.

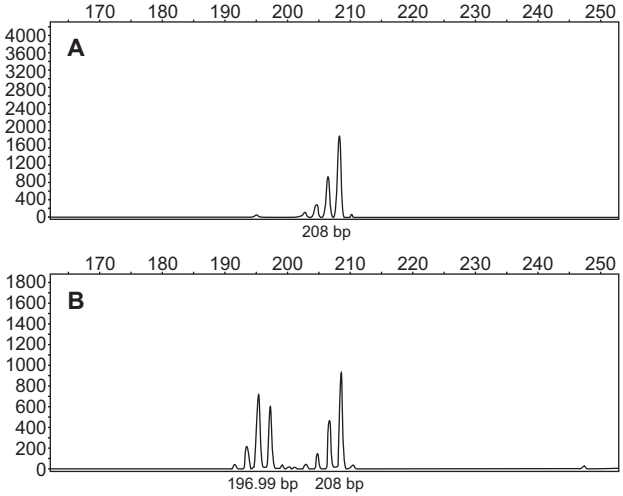

Figure 2. MSI of D2S123 marker in a homozygous patient with CRC, demonstrated by deletion of 11bp: there are two PCR products from tumor tissue with a length of 208 bp and 197 bp, respectively **(B)**, whereas in the paired normal tissue **(A)** there is only one PCR product with a length of 208 bp.

Methylation-specific PCR (MSP)

DNA methylation patterns in the promoter CpG islands of *MLH1* and *MSH2* genes were determined only in patients with MSI/LOH tumors applying MSP. This method distinguishes unmethylated from methylated alleles of a given gene [26,27]. After bisulfite

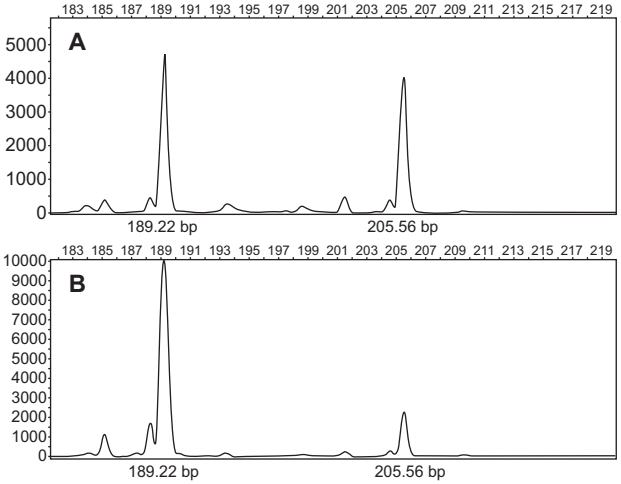

Figure 3. LOH of FGA marker in a heterozygous patient with CRC demonstrated by a significant reduction of the area under the peak of the longer allele of 206 bp in the tumor tissue **(B)** compared to that in the paired normal tissue **(A)**, whereas the peaks of the shorter allele of 189 bp were comparable in both tissues.

treatment of DNA, which converts unmethylated but not methylated cytosine to uracil and leaves 5-methyl cytosine unchanged, DNA was amplified by PCR using primers specific to methylated and unmethylated sequences. The bisulfite modification of DNA was performed using a MethylCode Bisulfite Conversion Kit (InvitrogenCorporation, Carlsbad, CA, USA).

The following set of primers was used for *MLH1* promoter methylation status: *MLH1msp_MF* (5′-GAT AGC GAT TTT TAA CGC-3′) (sense) and *MLH1msp_MR* (5′-TCT ATA AAT TAC TAA ATC TCT TCG-3') (antisense) for methylated reaction of *MLH1* and *MLH1msp_UF* (5′-AGA GTG GAT AGT GAT TTT TAA TGT-3′) (sense) and *MLH1msp_UR* (5′-ACT CTA TAA ATT ACT AAA TCT CTT CA-3') (antisense) for unmethylated reaction of *MLH1* [28,29].

For determination of promoter methylation of *MSH2* we applied two sets of primers flanking two different *MSH2* promoter regions provisionally named by us "msp1" and "msp2" regions:

MSH2msp1_UF (5′-GGT TGT TGT GGT TGG ATG TTG TTT-3′) (sense) and *MSH2msp1_UR* (5′-CAA CTA CAA CAT CTC CTT CAA CTA CAC CA-3′) (antisense) for unmethylated reaction of "msp1" region of *MSH2* and *MSH2msp1_MF* (5′-TCG TGG TCG GAC GTC GTT C-3′) (sense) and *MSH2msp1_MR* (5′-CAA CGT CTC CTT CGA CTA CAC CG-3′) (antisense) for methylated reaction [27];

MSH2msp2_UF (5′-TTG GTG ATT TTA GTT ATT GTG TAT GTT TGT-3′) (sense) and *MSH2msp2_UR* (5′-ATA AAC CCA TAA TCC CAA TAT TAA CAA TTT-3′) (antisense) for unmethylated reaction of "msp2" region of *MSH2* and *MSH2msp2_MF* (5′-TTT AGT TAT TGC GTA TGT TTG CGT TTA-3′) (sense) and *MSH2msp2_MR* (5′-CCG TAA TCC CGA TAT TAA CAA TTT ACT T-3′) (antisense) for methylated reaction [30].

The MSR products of the methylated reactions of *MLH1* were with a size of 91 bp and those of unmethylated reactions – 103 bp. The MSP products of the unmethylated reactions of "msp1" region of *MSH2* were 92 bp in length and of the methylated reaction – 76 bp in length; respectively, the MSP products of "msp2" region were 95 unmethylated and 90 bp of methylated reaction. The products of MSP reactions were detected on 2% agarose gel electrophoreses with ethidium bromide.

Statistical analyses

Statistical analyses were performed using StatView™ v.4.53. for Windows (Abacus Concepts, Inc.). Descriptive statistical tests, including mean, median and standard deviation were calculated according to the standard methods. The frequencies of distribution in contingency tables were analyzed using x^2 test and Fisher's exact test. For the outcome analysis, patients were classified according to the presence of MSI/LOH (high and low together), LOH and those without any of the microsatellite alterations (MSS). The primary outcome was overall survival, which was defined as the time from study entry (surgical therapy) to death or end of the follow-up period. Survival curves were generated according to the Kaplan-Meier method and the difference between the curves was assessed with the use of log-rank test. Factors with $p<0.05$ were considered statistically significant.

Results

Microsatellite instability

We extracted genomic DNA of patients with sporadic CRC both from archival paraffin-embedded and fresh frozen biopsies of tumor tissue and the corresponding normal mucosa. As it was expected, those DNA samples obtained from paraffin-embedded tissues appeared to be with less quality compared to the genomic DNA obtained from fresh frozen samples. Nevertheless, the quality and quantity of the DNA from paraffin-embedded samples provided a good enough quantity of specific PCR products for fragment analyses for assessing MSI in 74 patients. Thus, in addition to the 21 patients with tumor biopsies only, we lost another 18 patients (18/92) for the further analyses because of the poor quality of DNA extracted from the paraffin-embedded sections.

Finally, successful amplification of the DNA template from paired normal and tumor tissues was performed for 126 patients. Among them 36 patients (28.6%) appeared to have some alterations in one or more MSI markers: 6 patients had tumor with a length change in the normal alleles of one MSI marker (considered as MSI-L); 14 patients had tumors with length changes in the normal alleles of 2 or more MSI markers (considered as MSI-H) (Figures 1 and 2); 11 patients had tumors with LOH in one or more microsatellite markers (Figure 3); and 5 patients were with tumors with both alterations, length changes in normal alleles and LOH of microsatellite markers (one patients with MSI-l/LOH and 4 patients with MSI-H/LOH). In further analyses, patients with no alterations in any of the studied MSI markers (MSS, n=90) were compared with: i) the whole group of patients with any change in the microsatellite markers, MSI or/and LOH (MSI/LOH, n=36); ii) the subgroup of patients with length changes of any microsatellite marker, including those with MSI and LOH simultaneously (MSI, n=25); iii) the subgroup of patients with LOH of any microsatellite marker, including also those with MSI and LOH simultaneously (n=16).

MLH1 and MSH2 promoter hypermethylation

The methylation-specific PCR for detection of methylation status of *MLH1* and *MSH2* promoter regions was performed with genomic DNA extracted from tumor tissue of 30 patients showed MSI/LOH. For 6 of the patients with MSI/LOH tumors the quantity of genomic DNA was insufficient for bisufite modification and further MSP. From those 30 patients promoter hypermethylation of MLH1 was found in 15 cases (50%) (Figure 4).

Hypermethylation was observed more often in *MSI-H* tumors – 8 cases (53%) (7 with MSI-H and one case with MSI-H/LOH). *MLH1* promoter hypermethylation was also a frequent event in tumors with LOH only (5 cases, 33%). In addition *MLH1* hypermethylation was detected in another 2 MSI-L cases.

The MSP analyses for *MSH2* promoter hypermethylation showed a partial methylation in "smp1" region of one case (No.372), while the MSP for the "msp2" region detected full methylation in the same case (Figure 5).

That patient had a tumor with high microsatellite instability (MSI-H), defined as having alleles with length alterations in 4 of the studied microsatellite markers: D5S346, D2S123, D18S35 and FGA and LOH of D18S35 (Figure 6).

MSI and clinical presentation of CRC

No difference in the frequency of occurrence of MSI/LOH between genders was found: 24.4% of the males vs. 35.4% of females (x^2, p=0.182). However, there was a tendency for more frequent presence of length alterations (only MSI) in women (31.1%) compared to men (15.7%) (x^2, p=0.053). No difference was found when the presence of LOH was compared only between males and females (13.2% in males vs. 16.2% in females, x^2, p=0.677).

Patients with tumors with any alterations in the studied MSI markers (MSI/LOH) were more often younger than 60 years compared to the patients with MSS tumors (50 vs. 22%, x^2, p=0.002) (Figure 7). The

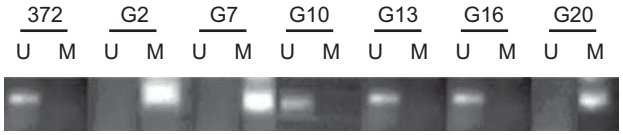

Figure 4. Analyses of methylation of promoter regions of *MLH1* in tumors of CRC patients with MSI/LOH.

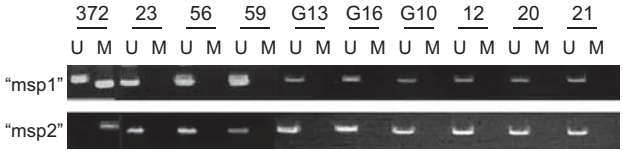

Figure 5. Analyses of methylation of "msp1" and "msp2" promoter regions of *MSH2* in tumors of CRC patients with MSI/LOH.

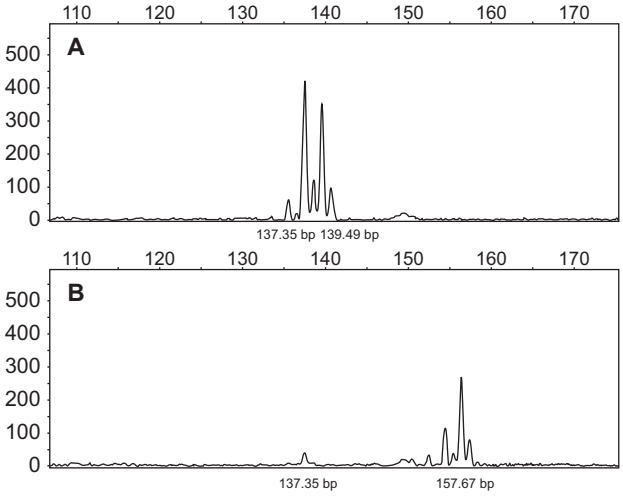

Figure 6. MSI and LOH of D18S35 marker in a heterozygous patient (No 372) with CRC. LOH is demonstrated by a significant reduction of the area under the peak of the shorter allele of 137.35 bp in the tumor tissue **(B)** compared to that in the paired normal tissue **(A).** The MSI is demonstrated with a presence of 18 bp longer allele with a length of 157.67 bp in tumor tissues **(B)** compared to the allele of 139.49 bp in the paired normal tissue **(A).**

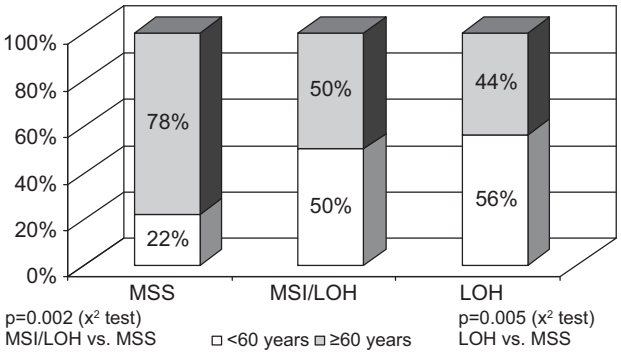

Figure 7. Association of the presence or MSI and/or LOH with the age of the patients with CRC.

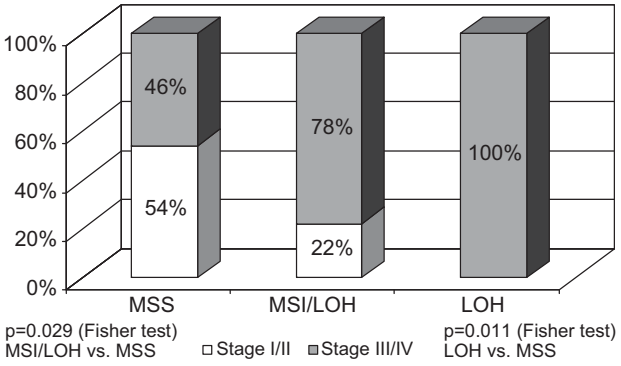

Figure 8. Association of the presence or MSI and/or LOH with tumor stage.

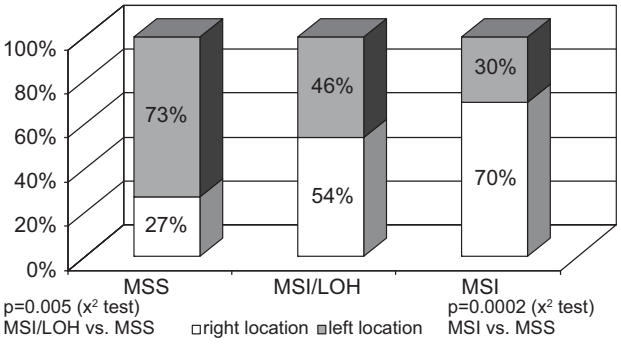

Figure 9. Association of the presence or MSI and/or LOH of MSI markers with tumor localization.

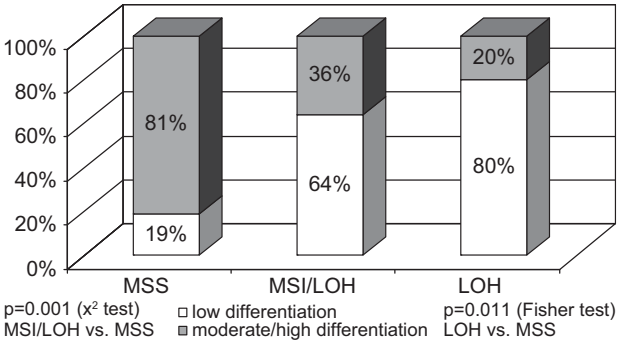

Figure 10. Association of the presence or MSI and/or LOH of MSI markers with tumor differentiation grade.

presence of MSI and/or LOH was associated with more advanced stage of the primary tumors (stage III/IV) (78 vs. 46%, Fisher's exact test, p=0.029, Figure 8), with right-sided tumor localization (55 vs. 27%, x^2, p=0.005, Figure 9) and lower degree of differentiation (64 vs. 19%, x^2, p=0.001, Figure 10) compared to the absence of any alterations of the studied microsatellite markers (MSS). The right-side localization was even more frequent when only patients with MSI were compared to those with MSS tumors (70 vs. 27%, x^2, p=0.0002) (Figure 9). No difference was found between patients with tumors with LOH and those with MSS (27 vs. 27%, Fisher's exact test, p>0.999). However, the differences in the frequencies of younger age, advanced stages and lower degree of differentiation were bigger when only patients with LOH were compared to those with MSS tumors (56 vs. 22%, p=0.005 for age; 100 vs. 46%, p=0.011 for stages; 80 vs. 19%, p=0.011 for degree of differentiation, Fisher's exact test) (Figures 7, 8 and 10).

Clinical records with survival data were available for 62 of the patients studied for MSI. The median overall survival was 31.7 months, ranging from 0.2 to 110 months. At the end of the follow-up period 29 (47%) patients were alive with a median overall survival of 48.7 months (range 27-110), whereas 33 of the patients (53%) had died. They had a median overall survival only of 16.4 months (range 0.2 – 49). Patients having tumors with MSI/LOH (n=12 with available survival data) appeared to have no significant difference in overall survival compared with those without any alterations in the studied microsatellite markers (log-rank, p=0.566) (Figure 11a) independently of tumor stage (log-rank, p=0.707) (Figure 11b).

Discussion

In this work we analysed the changes in 5 microsatellite markers in sporadic CRCs compared to the normal colorectal mucosa of the same patients or to other unaffected normal cells (blood cells) Appearance of such changes, deletions or insertions of the nucleotide repeats of the studied microsatellites in tumor genomic DNA compared to the genomic DNA from non-affected tissues are markers for the presence of MSI. The obtained frequency (22%) of MSI (either low or high MSI, 25 out of 115 patients) in our cohort of Bulgarian patients with sporadic CRC was commensurable to some other studies [31] and relatively higher than others, describing occurrence of MMR deficiency in about 15% of sporadic CRC [2,8,11]. It is quite possible that our observed frequency of MSI may not be representative

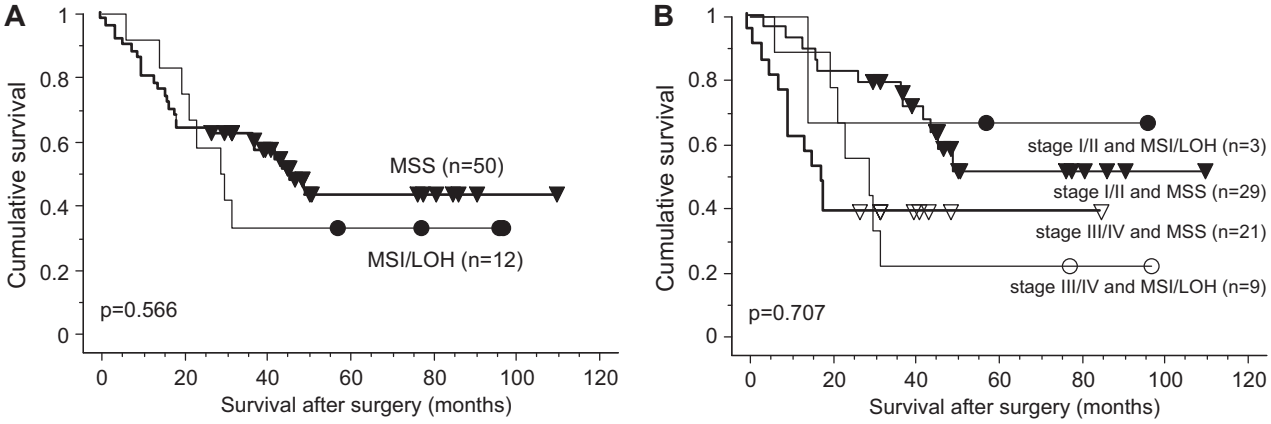

Figure 11. Overall survival of the patients according to the presence of MSI/LOH or MSS **(A)** and after stratification according to stage **(B)**.

of the frequency of MSI across Bulgarian CRC patients because our cases were only from two institutions and were not consecutive referrals.

The decreased level (more than 50%) of the amplification product of one or more alleles of microsatellite markers in tumor tissue compared to the normal tissue was considered as LOH. Such changes were seen either independently (in 11 patients) or concomitantly with length allele changes (MSI) (in 5 patients). This observation is in line with the notion that the complex process of carcinogenesis involves different mechanisms for functional changes of key regulatory genes [2,8,9].

In the cohort of patients presented in this report the MSI and/or LOH were more frequent events in younger patients, and these genetic changes were associated with advanced stages, poor differentiation and right-sided tumor location. These findings are in line with some of the results reported in several other studies. Thus Chang et al. have described that MSI-H tumors of Chinese patients with CRC were associated with young age, high carcinoembryonic antigen (CEA) level, right-sided tumor, poorly differentiated, mucin production, high *BRAF* mutation, lower allelic loss and relatively good prognosis [32]. Benatti et al. have also reported that MSI-H were more frequently in right-sided, poorly differentiated, with mucinous phenotype than in microsatellite stable carcinomas, but they have found that MSI tumors were detected more often at a less advanced stage, which differs from our observation [21]. In our study we also observed that cases with LOH of some of the microsatellite markers, although less frequent than those with MSI in sporadic CRC, were more markedly associated with younger age at diagnosis, lower degree of differentiation and advanced stages. These findings are partially in accordance with another report showing that LOH, particularly at *hMLH1* and *hPMS1* loci, even though not associated with clinicopathological parameters, such as Dukes' stage and tumor differentiation, was more frequent amongst the younger patients (< 50 years) [33].

There are many studies evaluating the role of MSI as a prognostic marker and predictor of response to chemotherapy in CRC, with inconclusive results. A number of studies along with one meta-analysis have shown a better prognosis of patients with MSI-H tumors when compared to MSS tumors with respect to overall and disease-free survival [20,34-36]. However, other studies found no influence of MSI on survival [19,37,38]. We also did not find association of MSI/LOH with the prognosis of the patients. We considered the possibility that our results might have been influenced by the relatively small number of patents in the group eligible for survival analyses and probably by the higher proportion of advanced tumors: 23% stage III and especially stage IV – 31%. A future study would require an increased number of patients to further support the current results.

It is well established that the presence of MSI is due to the failure of DNA MMR system, which leads to length changes of microsatellite sequences (MSs). Germline mutations in the genes encoding some of the mismatch repair enzymes are the cause of the HNPCC. The most commonly mutated MMR genes are *MLH1, MSH2* and *MSH6* [8-10]. In tumors with MSI from patients with no obvious family history, mutations of MMR genes are rare. LOH is one of the mechanisms for gene inactivation, and previous studies have detected LOH at the MLH1 region in HNPCC tumors [39].

Besides point mutations and LOH, DNA methylation is also a well-known cause of inactivation of MMR system. This mechanism is especially important in sporadic CRCs. Several studies indicated an inverse correlation between DNA methylation in the promoter

region of some of the MMR genes (*MLH1* and *MSH2*) and tissue-specific expression of the protein molecules [27,31,40] or mRNA [41]. Therefore, DNA hypermethylation of MMR genes, particularly *MLH1* and possibly *MSH2*, provides a potential mechanism to inactivate the gene expression and MMR system during colorectal tumorigenesis.

In our study we found that half of the studied tumors with MSI/LOH had promoter hypermethylation of *MLH1*, and only one of *MSH2* gene, as notably the patients with hypermethylation in *MLH1* or *MSH2* genes had predominantly MSI-H tumors. Our results correspond to those from other research groups, describing DNA methylation in proximal (5') regions of *MLH1* as a frequent event in sporadic CRCs with MSI [27,31], whereas hypermethylation of the promoter region of *MSH2* was not found [27], or detected in a low proportion of MSI tumors [40]. The failure of MMR system in other tumors with MSI/LOH is possibly a result of other mechanisms.

Recently, in addition to the DNA hypermethylation, other epigenetic mechanisms for gene expression modulation have been proposed. These include histone modification and various RNA-mediated processes [12,15,42], such as miRNAs that can regulate gene expression inducing direct mRNA degradation or translational inhibition [43]. Lately, it has been found that the relative expression of 6 miRNAs (miR-92, -223, -155, -196a, -31, and -26b) was significantly different among MSI subgroups, and miR-31 and miR-223 were overexpressed in CRCs of patients with HNPCC. These findings indicated that miRNA expression in CRC is associated with MSI subgroups, including low MSI-associated cancers and HNPCC, and suggest that miRNAs may have posttranscriptional gene regulatory roles in these MSI subgroups and possible consequences on the clinicopathologic and biomarker characteristics [16].

In conclusion, our data allow the suggestion that sporadic CRCs with microsatellite alterations (MSI and/or LOH), are more aggressive, develop earlier and progress faster to more advanced stage. The most frequent cause for the failure of DNA mismatch repair system was hypermethylation of CpG islands of the promoter region of *MLH1*, whereas the promoter methylation of *MSH2* was a rare event.

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