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hMLH1 and hMSH2 Somatic Inactivation Mechanisms in Sporadic Colorectal Cancer Patients

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Much is known about the role of germline inactivation in mismatch repair (MMR) genes in hereditary non-polyposis colorectal cancer (HNPCC), but the impact of somatic MMR gene changes on sporadic colorectal cancer remains to be elucidated. In hereditary cases the hMLH1 and hMSH2 genes were shown to have a great importance, and in order to examine the somatic inactivation mechanisms of the two MMR genes hMLH1 and hMSH2 we screened 37 Hungarian sporadic colorectal cancer patients for allelic imbalance (AI), microsatellite instability (MSI), hMLH1 promoter hypermethylation and somatic mutations. Thirteen of the examined

tumours (35%) were characterized by low-level MSI and none of the cases belonged to the high MSI group. Nine (24%) and seven (19%) cases had AI at the hMLH1 and hMSH2 genes, respectively. Seven tumours (19%) showed dense promoter hypermethylation of hMLH1, but only two patients had somatic mutations, one for each MMR gene. According to our study on this limited set of cases the most prominent mismatch repair inactivation mechanism in sporadic colorectal cancer patients is the hMLH1 promoter hypermethylation which may have a role in the carcinogenesis of sporadic colorectal cancer. (Pathology Oncology Research Vol 9, No 4, 236–241)

Keywords: sporadic colorectal cancer, mismatch repair, hMLH1, hMSH2, microsatellite instability, allelic imbalance, promoter hypermethylation, and inactivation

Introduction

In Hungary, colorectal cancer is the third most frequent malignant disease, with an annual incidence of 70 cases/100,000 inhabitants. Hereditary cancer accounts for 5-10% of all colorectal cancers and two thirds of the familial cases are diagnosed with hereditary non-polyposis colorectal cancer (HNPCC)¹. The establishment of the genomic instability is a necessary and early step in the formation of human cancers increasing the mutation rate of the affected cells, thus predisposing them to the accumulation of genetic alterations. The mutator phenotype can be generated by several classes of genes; one of these classes includes genes encoding DNA mismatch repair proteins.³

The vast majority of HNPCCs were linked to the two main mismatch repair (MMR) genes hMSH2 and hMLH1 located on chromosome arms 2p and 3p, respectively.^{4,7} The inactivation of these genes, usually by point mutation,

enables tumours to accumulate mutations leading to replication error (RER) phenotype.^{3,8} The presence of cancer RER does not necessarily define which MMR gene is involved.

Beside point mutations, DNA methylation is also a well-known cause of the inactivation of HNPCC and sporadic colorectal cancer with microsatellite instability.⁹ Several studies indicated an inverse correlation between DNA methylation and tissue-specific expression of certain genes such as hMLH1.^{10,11,12,16,17,20,21} Therefore, DNA methylation provides a potential mechanism to inactivate specific genes during tumorigenesis, gene silencing by methylation can provide a selective advantage for cell growth. The methylation status of hMLH1 in microsatellite instability-low (MSI-L) and microsatellite stable tumours is not well defined. Hypermethylation of hMSH2 promoter region in colorectal cancer was not found recently.¹⁰⁻¹²

The aim of this study was to determine the frequency of microsatellite instability and to analyse the inactivation mechanisms of the mismatch repair genes in sporadic colorectal cancers in Hungary as it is well described in HNPCC cases and to define these mechanisms in different MSI groups.

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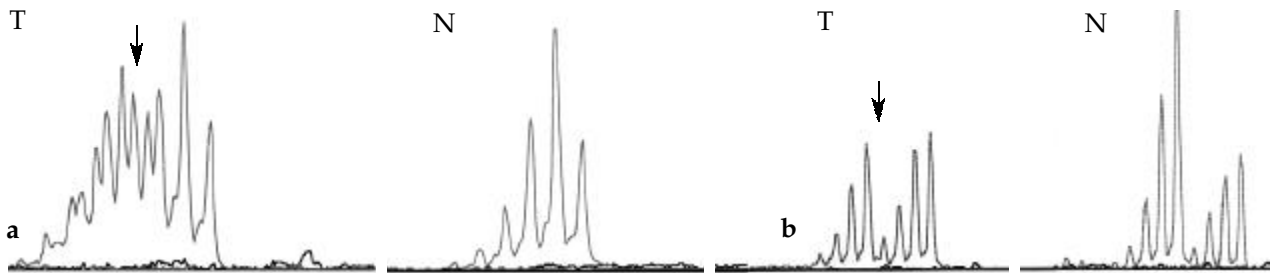


Figure 1. Microsatellite analysis near the hMSH2 gene by the D3S1283 marker, both tumour and normal samples were analysed. **a:** MSI phenotype in patient HuCRC-69, arrow indicates new allele appearing in tumour sample. **b:** AI observed in patient HuCRC-55, arrow indicates the allele with reduced intensity.

Materials and Methods

Patients, tissue and DNA samples 37 patients with colorectal carcinomas were selected at the National Institute of Oncology, Budapest, Hungary on the basis of negative family history of any type of cancer. Formal consents were obtained from the patients. The mean age of onset was 65 years (between 31 and 87 years). The individuals who had developed colon cancer were 19 males and 18 females with tumours of different Dukes' classifications: 1 A, 19 B, and 17 C. Cancerous and corresponding normal tissues of the patients were selected and isolated by an experienced pathologist. DNA was extracted after proteinase K (Sigma, St. Louis, MO, USA) digestion according to the standard phenol-chloroform protocol.¹³

Microsatellite analysis. Paired tumour and normal DNA was used for testing microsatellite instability (MSI), or allelic imbalance (AI). In AI we considered a threshold of 50% reduction as significant.¹⁴ Primer sequences were from GeneBank; the primers for (CA)_n repeats (D2S118, D2S123, D3S1283 (Figure 1), D3S1298, D3S1611, D5S346 and D16S398) and A_n (BAT26) repeat were fluorescently labelled. Analysis was performed using ABIPRISM 310 genetic analyser, with GeneScan software (Applied Biosystems, Foster City, CA). The MSI status was assessed according to the consensus of the National Cancer Institute Workshop on Microsatellite Instability for Colorectal Cancer Detection:¹⁵ high level instability (MSI-H) was diagnosed when more than 30% of the examined markers carried instability, low level instability (MSI-L) when less than 30% of the markers presented new alleles, and microsatellite stable phenotype (MSS) where no instability appeared in any of the markers examined. If microsatellite instability occurred at any locus, the result for this locus was considered as non-informative for the AI analysis.¹⁵

hMLH1 promoter methylation assay. The assay was performed as it was described earlier by Kane¹⁶ with inner controls. An unmodified PCR product was used as non-

methylated inner control and an SssI methylase-treated (New England BioLabs Inc, Beverly MA) PCR product as a positive control. The control primer pair amplifies the Mucl gene. A volume of 500 ng of genomic DNA and, in parallel, the non-methylated and the methylated control DNA was digested with HpaII (Pharmacia Biotech Inc, Uppsala, Sweden) and the template DNA with the non-methylated control DNA was digested with MspI (Promega Corporation, Madison, WI, USA) restriction endonucleases in a 20 µl volume according to the manufacturers' instructions. Ten microliters of the digests were analysed by PCR in 50 µl reaction volume as described by Kane.¹⁶ The control Mucl primer pair for the control DNA was: sense: 5'-accaagactgatgccagtagcact-3', antisense: 5'-accgttacctgcagaaaccttct-3'. The resulting amplification products were analysed by 7.5% acrylamide gel using standard methods (Figure 2).

PCR reactions and Single Strand Conformation Polymorphism (SSCP). All exons from the hMLH1 and hMSH2 genes were analysed. Primers sequences and cycling conditions were used as published earlier.^{17,18} After sample denaturation gel electrophoresis was performed on a 16-cm long vertical slab gel (Hoefer SE 600

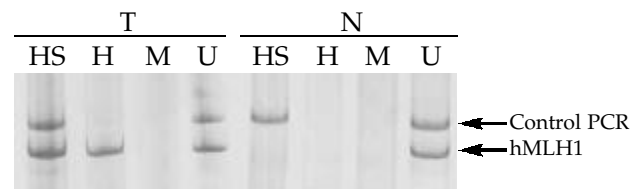


Figure 2. Examples for methylation assay of the hMLH1 promoter region in patient HuCRC-24. Amplification of the hMLH1 promoter region in tumour (T) and normal (N) samples, with or without treatment with the indicated restriction endonucleases. HS: containing hypermethylated control DNA (treated with SssI methylase), H: digested with HpaII, M: digested with MspI, U: undigested control. The presence of a visible PCR product in lane H indicates a densely methylated hMLH1 promoter in the DNA sample.

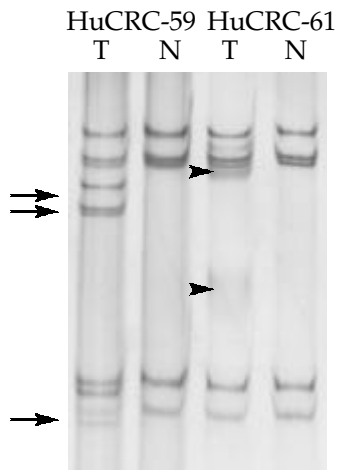


Figure 3. Mutation detection of exon 6 in the hMSH2 gene using SSCP. Mutation detection of exon 6 of the hMSH2 gene. PCR products from colon tumours (T) and normal (N) tissues were analysed. The extra bands indicated by arrows determine mutations in the representing tumour samples comparing to the non-mutated normal samples.

Series, Pharmacia Biotech Inc., San Francisco, CA, USA). Gels were stained using the silver staining protocol (Figure 3).

DNA sequencing. DNA sequencing of the purified PCR products showing altered migration patterns was performed by ABI-PRISM 310 genetic analyser using BigDye terminator cycle sequencing kit (Applied Biosystems).

Statistics. Comparison of different groups was performed using Fisher's exact test. Differences were considered significant when the p value was less than 0.05.

Results

Frequency of microsatellite instability and allelic imbalance. None of the patients showed the microsatellite unstable MSI-H phenotype (characterised by instability at >30% of the markers used). Thirteen of the 37 patients (35%) exhibited the MSI-L phenotype while the rest of the patients (65%) belonged to the MSS group. Allelic imbalance was found either near the hMLH1 locus (24%, 9/37), or in the hMSH2 locus affecting 19% (7/37) of the cases. The microsatellite status of colorectal cancer patients are summarised in Table 1.

Methylation of the hMLH1 promoter region. The presence of hMLH1 promoter hypermethylation was observed in 7/37 tumours (19%) (Table 1). The resistance to digestion by HpaII means hypermethylation at all four HpaII sensitive CpG islands in the promoter region of the gene

that is, dense hypermethylation of the promoter (Figure 2). None of the normal tissue samples tested exhibited hMLH1 promoter methylation.

Frequency of mutations. SSCP analysis of hMLH1 and hMSH2, followed by direct sequencing, revealed the presence of seven distinct somatic alterations of the MMR genes in our panel of sporadic colorectal carcinomas. Only two of these alterations were true pathogenic mutations, Lys618Thr in exon 16 of hMLH1 and Asp322Gly in the sixth exon of hMSH2, both described earlier (Table 2). Four of the alterations were silent polymorphisms or intronic variants, two in hMLH1 and two in hMSH2. The remaining alteration, Arg552Trp in exon 14 of hMLH1, is an unclassified sequence variant, as it has not been described earlier and its functional significance cannot be assessed without further studies (Table 2).

Germline mutations have also been found in the colorectal cancer patients analysed. In the hMLH1 gene a rare polymorphism occurred: IVS14-19A>G. In exon 8 a 655A>G polymorphism was observed causing Ile219Val. In exon 5 the polymorphism was an 837C>T change, which causes no change at protein level, because both code a leucine amino acid at codon 279. The last alteration has not been described earlier.

Discussion

Fidelity of DNA replication is crucial in avoiding the accumulation of mutations in the genes. hMLH1 and hMSH2 are members of post-replication mismatch repair genes. Functional disruption of these genes increases the rate of spontaneous mutations resulting in a mutator phenotype. As the MMR genes are recessive, both of the alleles should be inactivated for cancer development, by two independent events. These events can be allelic imbalance, promoter hypermethylation or mutations.

In this study 37 sporadic colorectal tumours were analysed to evaluate the frequency of the inactivation of these MMR genes. Altogether, twenty patients of the 37 (54%) had somatic inactivation in at least one of the MMR genes. We found no association between AI at any locus, MSI, promoter hypermethylation of the hMLH1 gene and clinical data (age or Dukes' stage). We examined 8 microsatellite loci, 3 on the 2nd chromosome near hMSH2 gene, and 3 on the 3rd chromosome near hMLH1 gene and two others on other chromosomes. None of the tumours showed MSI-H status, 35% of the cases was MSI-L, while the majority was microsatellite stable. The microsatellite instability is a less frequent event as described in the HNPCC patients. Other studies found different percentage of MSI-H cases in sporadic colorectal cancer patients, but the cause of the difference could well be that our criteria for the patient selection were very strict which did not

allow any family history of cancer. A potential cause of the lack of correlation between MSI status and MMR variants is that alterations in other MMR genes, such as hPMS1, hPMS2, hMSH6, hMSH3 or genes as yet unidentified might be involved. Other studies suggest that hMSH6 mutations are associated with MSI-L phenotype,¹⁹ however, the genetic basis of the MSI-L phenotype is still unknown. The AI was observed with about the same frequency in the hMLH1 and hMSH2 genes (24% and 19%). The allelic imbalance occurs at about the same frequency at the hMLH1 gene as the promoter hypermethylation, but

its effect for the inactivation is not well defined,¹⁴ as it can well be a second hit in the inactivation mechanism, but it could be a sign of the imbalance of another gene near the MMR gene on the chromosome or it may not have a role in the inactivation.

Hypermethylation of the hMLH1 gene was found in 19% of the colon cancers. Our hypermethylation analysis can underestimate the inactivation of the gene, as it is positive only when all four examined CpG islands of the hMLH1 promoter region are hypermethylated, although hemimethylation can also be sufficient for gene inactivation.²⁰ Other studies

Table 1. MMR inactivation with clinical data

Patient ID	AI ¹		MSI-L ²	hMLH1 promoter methylation	Mutation	aGE AT diagnosis (years)	Dukes' stage
	MLH1	MSH2					
HuCRC-5	-	-	+	-	-	64	C2
HuCRC-7	+	-	-	-	-	76	B2
HuCRC-8	-	+	-	-	-	70	C2
HuCRC-9	-	+	-	-	-	44	B1
HuCRC-12	+	-	-	+	-	66	C
HuCRC-14	-	-	+	+	-	87	C
HuCRC-15	-	-	-	+	-	67	B2
HuCRC-16	-	+	-	-	-	51	C
HuCRC-18	-	+	+	-	-	61	C2
HuCRC-19	-	-	-	-	-	79	B1
HuCRC-22	+	-	-	-	-	54	B
HuCRC-23	+	-	-	-	-	69	C
HuCRC-24	-	+	+	+	-	64	B2
HuCRC-26	-	-	-	-	-	60	B2
HuCRC-28	-	-	-	-	-	50	C2
HuCRC-29	-	-	+	+	-	48	B
HuCRC-36	+	-	-	-	-	85	C2
HuCRC-39	-	-	-	-	-	76	B2
HuCRC-42	+	-	-	-	-	69	C3
HuCRC-43	+	+	+	-	-	78	B1
HuCRC-45	-	-	-	-	-	64	A
HuCRC-46	-	-	-	-	-	71	C2
HuCRC-48	-	-	-	-	-	72	B2
HuCRC-49	-	-	+	-	-	71	C
HuCRC-50	-	-	+	-	-	62	C2
HuCRC-53	-	-	-	+	hMLH1	31	B2
HuCRC-54	-	-	+	-	-	77	B2
HuCRC-55	+	-	-	-	-	40	B
HuCRC-59	-	-	-	-	hMSH2	64	B2
HuCRC-60	-	-	-	-	-	47	B2
HuCRC-61	-	-	-	-	-	59	C1
HuCRC-63	-	-	+	-	-	76	C
HuCRC-64	-	-	-	-	-	56	B1
HuCRC-65	-	+	+	+	-	75	B1
HuCRC-67	-	-	-	-	-	83	C
HuCRC-69	-	-	+	-	-	71	C2
HuCRC-70	+	-	+	-	-	68	B

1: Allelic imbalance at the nearest informative locus.

2: There was no MSI-H in this set of patients.

Table 2. Somatic hMLH1 and hMSH2 alterations

Gene	Exon	Nucleotide change	Predicted protein change	Patient code	Described earlier
<i>Mutation</i>					
HMLH1	16	1535A>C	Lys618Thr	HuCRC-53	(25)
HMSH2	6	965A>G	Asp322Gly	HuCRC-59	(26, 27)
<i>Polymorphism</i>					
HMLH1	17	1959G>T	none (Leu653Leu)	HuCRC-70	(28, 27)
hMSH2	6	984C>T	none (Ala328Ala)	HuCRC-61	(27)
hMSH2	13	2061C>G	none (Leu687Leu)	HuCRC-29	Not described
hMLH1	9	790+10 A>G	none	HuCRC-19	(27)
<i>Not classified</i>					
hMLH1	14	1564C>T	Arg522Trp	HuCRC-9	Not described

suggest that only dense methylation of the hMLH1 promoter correlates with the decreased level of mRNA expression.²¹ Biallelic involvement is common on the basis of previous studies, and in this case, hypermethylation alone could explain almost all cases of extinct hMLH1 expression.²⁰

The mutational event was less frequent in our sporadic colorectal cancer patients. Two true somatic pathogenic mutations were observed, one of them in the hMLH1 and the other in the hMSH2 gene. Both of the patients had Dukes' B stage cancer. The patient with the hMLH1 mutation was the youngest of our study, she was only 31 years old at the age of onset, and carried also promoter hypermethylation of the same gene. The patient with the hMSH2 mutation was at the mean age of our patients which did not show other alterations of the MMR genes and did not carry either AI or MSI. Studies suggest that certain missense mutations of the MMR genes do not correlate with MSI, although they segregate with the disease, and inactivate the MMR system.²² From the four different somatic polymorphisms one on the hMSH2 gene (Leu687Leu) has not been described earlier. Three of eleven genetic alterations found in the Hungarian population have not been documented before (one germline, one somatic polymorphism and one unclassified alteration), while three other somatic alterations (one intronic, one polymorphism and one missense mutation) were found by other Central Eastern European groups in Poland, Russia and Slovakia, suggesting that the majority of these alterations are specific for these populations. Among the somatic alterations, there was an unclassified one, observed in exon 14 at codon 522, and we found it in a patient with Dukes' B2. This alteration might be a true pathogenic mutation altering an amino acid residue present from *Drosophila* to human, but the effect of this alteration is difficult to interpret, and further studies of its functional characterisation are underway. Although the pathogenic effects of a missense mutation may be difficult to reveal, analysing patients with sporadic colorectal cancers

may identify a subgroup of patients with distinct clinico-pathological features, which might respond differently to chemotherapy.²³

Our data suggest that mismatch repair gene methylation inactivation of hMLH1 in non-MSI-H sporadic colorectal cancer has about the same frequency as in its hereditary variants. Although in hereditary cancer inactivation of the hMSH2 and the hMLH1 genes are of much the same importance, in sporadic cancer hMLH1 gene has a significantly higher inactivation frequency which indicates that the involvement of the hMLH1 mismatch repair gene in the pathogenesis of the sporadic colorectal cancer is more common than it was previously thought.

In conclusion, these results indicate that the inactivation of the mismatch repair genes in sporadic colorectal carcinoma is at least as important as in the case of hereditary colorectal cancer. In addition, our data suggest that the principal mechanism of gene inactivation in the case of hMLH1 gene is promoter hypermethylation and this lends further support to the view that CpG island methylation is a distinct genetic profile of colorectal tumours.²⁴ Further studies on a larger set of colorectal patients can confirm these data described above.

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