Evaluation of Microsatellite Instability, MLH1 expression and hMLH1 promoter hypermethylation in colorectal carcinomas among Tunisians patients

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Evaluation de l'instabilité microsatellitaire, de l'expression de MLH1 et du status de méthylation du promoteur de hMLH1 dans une série tunisienne de cancers colorectaux

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RÉSUMÉ

Prérequis : 10 à 15% des cancers colorectaux sporadiques montrent une instabilité microsatellitaire. Cette dernière est généralement associée à une hyperméthylation du promoteur du gène de hMLH1.

But : Etudier la relation entre le phénotype MSI, l'expression de la protéine MLH1 et le statut de méthylation du promoteur du gène de hMLH1 dans notre série tunisienne de cancers colorectaux sporadiques.

Méthodes: Nous avons étudié l'expression de MLH1 et MSH2 par immunohistochimie, le phénotype MSI par électrophorèse automatisée et le statut de méthylation du promoteur du gène de hMLH1 par méthylation-specific PCR.

Résultats: 57% des cas sont MSS, 28% sont MSI-L et 15% sont MSI-H. Ces derniers montrent fréquemment une localisation droite, un stade III TNM, et sont majoritairement mucineux. La majorité des cas MSS/MSI-L (79%) sont non-méthylés contrairement aux cas MSI-H (26%). 84% des cas MSS/MSI-L expriment positivement MLH1et 52% des cas MSI-H. Pour les cas MSI-H méthylés, 35% expriment la protéine MLH1 alors que 100% des cas non-méthylés sont positif immunohistochimiquement pour MLH1. Concernant les 11 cas MSI-H qui sont negatifs pour MLH1, ils présentent une méthylation du promoteur du gène de hMLH1. Cepandant 50% des cas MSI-H, positifs pour MLH1, sont méthylés.

Conclusion: Nos resultants montrent que la phenotype MSI-H présente majoritairement une localisation droite, un Stade III TNM et est de type mucineux. La relation entre l'expression de MLH1 et le statut de méthylation du promoteur de hMLH1 dans notre série Tunisienne est en corrélation avec les résultats d'autres séries de la littérature.

Mots-clés

MSI; MLH1; hyperméthylation ; carcinome colorectal.

SUMMARY

Background: About 10% to 15% of sporadic colorectal cancers demonstrate high level of microsatellite instability that is generally associated with aberrant methylation of hMLH1 promoter.

Aim: To investigate the association between MSI status, hMLH1 protein expression and methylation status of the hMLH1 promoter in a cohort of Tunisian sporadic colorectal cancer.

Methods: Expression of MLH1 and MSH2 was determined by immunohistochemestry and the MSI status was analysed by microfluid-based on-chip electrophoresis. Methylation of the hMLH1 gene promoter was determined by methylation-specific PCR.

Results: Of the 150 colorectal cancers 57% were MSS, 28% were MSI-L and 15%were MSI-H. MSI-H tumors were more frequently right-sided, exhibited a stage III of TNM and tended more to be mucinous. The MSI status had no effect on overall patient survival. Most of the MSS/MSI-L 79% cancers were unmethylated at the hMLH1 promoter, while 26% MSI-H cancers were unmethylated. 84% of MSS and MSI-L expressed MLH1 and 52% of MSI-H expressed MLH1. Of the methylated MSI-H cases, 35% expressed MLH1 protein while 100% of the unmethylated MSI-H were positive for MLH1 staining. Of 11 MSI-H cancers with loss of MLH1 expression, all cases were also methylated while 50% MSI-H cancers with positive immunostaining for MLH1 were methylated at the hMLH1 promoter.

Conclusion: Our study showed that MSI-H phenotype was mucinous, right-side and exhibit stade III of TNM. The relative correlation of MLH1 expression and promotor hypermethylation of hMLH1 for the MSI status is similar to that reported for several study.

Key-words

MSI; MLH1; promoter methylation; colorectal carcinoma.

Colorectal cancer (CRC) occupies the fourth rank among all types of cancers [1] and the first gastro-intestinal cancer by organ location [2, 3]. It constitutes an actual public health issue [3, 4]. The etiology of human cancer from a benign neoplasm to malignant tumor has been explained by pathways involving the accumulation of genetic and/or epigenetic alterations [5]. CRC pathogenesis proceeds through two well defined pathways of genomic instability, termed as the suppressor and mutator pathways.

These pathways are characterized by successive accumulation of genetic events in neoplastic cells as a function of time. The first mechanism is observed at the chromosomal level and leads to gene alterations by chromosomal gains and losses, accompanied by mutations at specific tumor suppressor genes and oncogenes [6]. The second mechanism was observed in size variations of short repetitive DNA sequences in tumor DNA, termed microsatellites [7-9]. Microsatellites, also known as variable nucleotide tandem repeats (VNTRs), are loci throughout the genome in which a short motif, such as a dinucleotide or trinucleotide sequence, is repeated at least several times.

During the process of DNA replication, defects in mismatch repair may lead to alterations in the lengths of microsatellites in the daughter cells. In a clonally expanding population of cells such as that of a tumor, the alteration may be carried forward to future generations of cells. In the laboratory setting, these changes are detected by comparing the length of a particular microsatellite allele in tumor DNA to that of a normal cell. This phenomenon is termed microsatellite instability (MSI) and has been reported to occur in almost all cases of colon cancer with hereditary non polyposis colorectal cancer (HNPCC) [10] and 10 to 20% of cases of sporadic colon cancer [11].

Among sporadic cases, the mechanism by which the instability occurs involves promoter hypermethylation of the DNA mismatch repair gene hMLH1 and silencing of its transcription [12]. In fact, patients with CRC tumors displaying MSI is also associated with distinct clinicopathologic features (e.g., proximal tumors site, high grade, early stage, diploidy, and favorable survival [13-17].

In 1997, in an attempt to provide uniformity in clinical diagnoses, an international meeting at the National Cancer Institute (NCI) recommended primary microsatellite markers for use in CRC MSI testing in clinical and research settings. The recommended Bethesda MSI testing set comprises the microsatellite loci Bat25, Bat26, D2S123, D5S346 and D17S250, characterised by mononucleotide and dinucleotide repeats [11]. The NCI also recommended the use of a reference panel of 5 DNA microsatellites to characterize a tumor's degree of MSI by using the terms "microsatellite stable" (MSS), "low frequency MSI" (MSI-L), and "high-frequency MSI" (MSI-H) [11].

In the current study, we intended to evaluate microsatellite instability and its relationship to clinicopathologic findings., hMLH1 expression and hMLH1 promoter hypermethylation in a cohort of Tunisian sporadic colorectal carcinoma patients.

MATERIAL AND METHODS

Patients

We performed a retrospective study from 1995 to 2009 concerning 150 patients with sporadic colorectal carcinoma, collected in the laboratory of Pathology of the Mongi Slim Hospital of Tunis. In this study, samples were taken not only from the tumoral area but also from margin, corresponding to distant resection, and were histologically free from pre-cancer and cancer. Furthermore, all tumors initially reported as mucinous carcinomas were reviewed by two pathologists and those in whom histological mucinous extracellular pattern was 50% or greater of their volume were included in the mucinous group.

DNA extraction

The genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue samples and frozen colorectal tissues. Tissue and deparaffinised sections were lysed overnight at 50°C by proteinase digestion (invitrogen). Then DNA was extracted from lysates of normal and tumor mucosa by means of the Wizard SV Genomic DNA Purification System according to the manufacturer's instructions (Promega, Madison, WI). The concentration of the DNA was measured with a spectrophotometer.

Detection of Microsatellite Instability

For amplification of microsatellite loci, which are recommended by Bethesda guidelines (mononucleotide repeats: Bat25 and Bat26; dinucleotide repeats: D2S123, D5S346 and D17S250), primers shown in table 1 were used.

Table 1: Characteristics of the microsatellite markers analyzed

Microsatellite	Locus	Primer Sequence (5' to 3')	Size [bp]	Tm*
Bat25	4p12	F : TCGCCTCCAAGAATGTAAGT R : TCTGCATTTTAACTATGGCTC	≈ 125	58°C
Bat26	2p	TGACTACTTTTGACTTCAGCC AACCATTCAACATTTTTAACCC	≈ 120	58°C
D2S123	2p16	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC	210-230	60°C
APC- D5S346	5q21/22	ACTCACTCTAGTGATAAATCG AGCAGATAAGACAGTATTACTGTT	110-130	54°C
MFd15 D17S250	17q11.2-q12	GGAAGAATCAAATAGACAAT GCTGGCCATATATATATTTAAACC	150-160	52°C

PCR was performed in 20μL reaction volumes using of 0.4 μmol of each primer, 0.5 mmol dNTPs, 1X PCR buffer (Promega), 1.5 mM MgCl2 (Promega), 0,04 units of Taqpolymerase (Promega). For the separation of microsatellite PCR products, we used DNA 1K Kits and the Experion Biorad bioanalyzer according to the manufacturer's instructions. In brief, the chips were prepared with gel-stain mix and then pressurized. Marker solution and DNA ladder were added. 1 μl of each PCR reaction was pipette into one of the twelve sample wells of the prepared chip. After vortexing, the chip was placed in the Experion bioanalyzer and run using the DNA 1K assay. Electrophoresis of the 12 samples took 30 - 40 minutes. Fragment analysis was carried out using Experion expert software. To identify MSI in the colorectal carcinoma patients an overlay of two electropherograms was used to compare PCR patterns derived from tumour and non-tumour tissues. Differences in the peak patterns of the overlaid electropherograms were evaluated. Tumors in which none of the loci showed instability were classified as MSS. Those with a single unstable locus were classified as MSI-L. Tumors with 2 or more unstable loci were classified as MSI-H.

Sodium bisulfite modification of DNA and methylation-specific PCR

Two micrograms of genomic DNA from each samples was bisulfite-modified using the EZ DNA methylation kit (ZYMO Research, Orange, CA) according to the manufacturer's instructions. After treatment, the resulting bisulfite-modified DNA was eluted in 10 μL of the kit elution buffer and stored at -20°C. Two microliter of the bisulfite-modified DNA was used for each PCR reaction. The following primers were used for MS-PCR analysis: for unmethylated (U) primers: ACCACCTCATCATAACTACCCACA (forward) and TTTTGATGTAGATGTTTTATTAGGGTTGT (reverse), and for methylated (M)-specific primers: ACGTAGACGTTTTATTAGGGTCGC (forward) and CCTCATCGTAACTACCCGCG (reverse).

Two microliter of bisulfite-modified DNA from each sample was amplified independently using the U- and M-specific primers in a 25 μL total volume reaction. Each PCR reaction contained a final concentration of 0.4 μmol of each primer (SGS, Köping, Sweden), 0.5 mmol dNTPs, 1X PCR buffer (Promega), 1.5 mM MgCl2 (Promega), 0,04 units of Taqpolymerase (Promega). The PCR-products were checked on a 3% agarose gel. The gel was briefly stained with 0.1 mg/mL ethidium bromide and viewed under UV light.

Immunohistochemical Analysis

The tissue samples have been taken systematically from tumoral lesion and from the areas between normal and tumoral tissue. They had been fixed routinely in 10% neutral formalin and embedded in paraffin. Sections were cut from each block, several of which were stained routinely for histological diagnosis including haematoxylin and eosin (HE), diastase-Periodic acid Schiff (d-PAS) and alcian blue staining. Two pathologists interpreted the results. Immunohistochemical analyses were performed including commercial available monoclonal antibodies to MLH1 (dilution 1:100, clone ES05, Novocastra, United Kingdom), and MSH2 (dilution 1:40, clone 25D12, Novocastra, United Kingdom). The sections were deparaffinized and rehydrated in graded alcohol. For heat-induced epitope retrieval, the sections were subjected to a 1.0-mmol/L concentration of citrate buffer (pH 6.0) (Novocastra) twice in microwave for 5 min each, then kept at room temperature for 20 min. The sections staining were performed using a Novocastra Concentrated Peroxidase Detection System RE7130-K, following the vendor's protocol. Two pathologists interpreted the results without knowledge of clinical and pathological information. To avoid artificial effect, cells in areas with necrosis, poor morphology or in the margins of sections were not counted. MLH1 and MSH2 proteins were considered positive in tissue samples exhibiting nuclear staining in > 10% of epithelial cells. The staining was closely compared between the samples of normal and tumoral mucosa from the same patient.

Statistical analysis

Statistical analysis was performed using the x2-test Fisher's exact test and cumulative observed overall survival rates were calculated using the Kaplan-Meier method using software developed by SPSS (version 17). The exact coefficient (P) for sample proportion analysis was performed to determine all significant parameters: differences were considered significant at the P-values less than 0.05.

RESULTS

The microsatellite loci of Bethesda panel, Bat25, Bat26, D2S123, D5S346 and D17S250, recommended for CRC analyses by the conference at National Cancer Institute [11], were amplified by label-free PCR. All five amplified microsatellite loci, including mononucleotide and dinucleotide repeats, were well resolved by microfluid-based on-chip electrophoresis Bio-Rad (figure 1). Of the 150 tumors from colorectal carcinomas, 85 (57%) were MSS, 42 (28%) were MSI-L and 23 (15%) were MSI-H. Previous research had indicated that MSS and MSI-L tumors have a common molecular background, [21] so comparisons were made between patients with MSI-H tumors and MSS or MSI-L tumors.

Clinical and pathologic characteristics of the patients according to MSI status are shown on Table 2. In our study, we did not find any correlation between MSI-H status, gender, age and growth pattern. MSI-H tumors were more frequently right-sided (p=0.0002) and they more often exhibited a stage III of TNM (p=0.007). About the histologigal type, MSS/MSI-L group tended more to be non-mucinous (p=0.002) whereas MSI-H has a slight predominance of mucinous type (13/23).

We also did survival analyses. Patients with both MSS and MSI-L status were compared with MSI-H status. The median follow-up time was 62 years for MSS/MSI-L and 72 years for MSI-H. In this cohort, the MSI status had no effect on overall patient survival (p=0.27) (figure 2).

Figure 1: Electropherograms of microfluidic-based separation of unlabelled PCR products representing each of the five microsatellite loci Bat25+D17S250, Bat26+D2123 and D5S346. (A) The patterns of the electropherograms representing PCR amplification products derived from normal tissue (red) and tumourous tissue (blue) are perfectly matching and demonstrate microsatellite stability. (B) The patterns of the electropherograms representing deviations in the electrophoretic patterns of the microsatellite loci: D17S250, D2123 and D5S346 indicating microsatellite instability (arrow indicate divergent pattern of peaks).

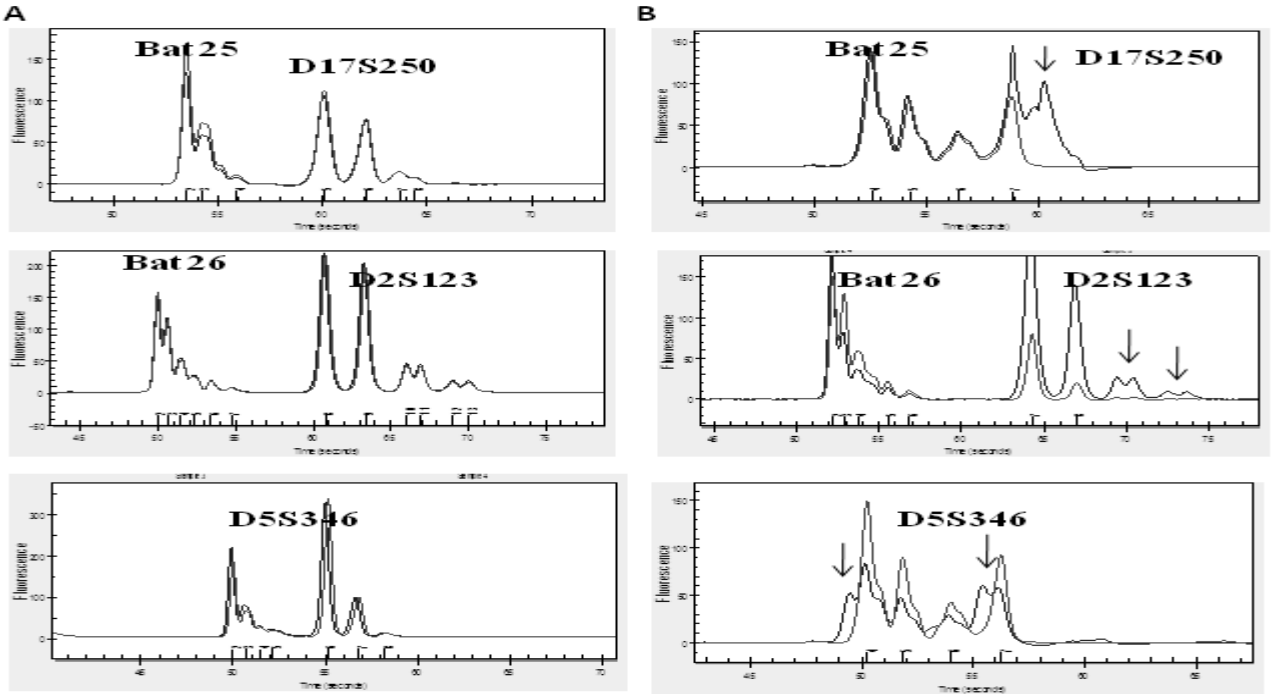
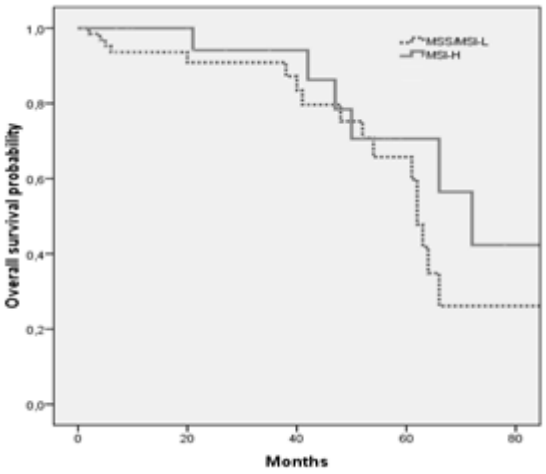


Table 2: Clinical and Pathologic Characteristics According to MSI Status

	MSS/MSI-L (%) n= 127	MSI-H (%) n= 23	P value
Sex			
Males	60 (47.2)	8 (34.8)	> 0.05
Females	67 (52.8)	15 (65.2)	
Age			
< 50	64 (50.3)	12 (52.2)	> 0.05
≥ 50	63 (49.7)	11 (47.8)	
Location			
Right Colon	47 (37)	18 (78.3)	0.0002
Left Colon	80 (63)	5 (21.7)	
Growth pattern			
Infiltrating	59 (46.4)	14 (60.9)	> 0.05
Expanding	68 (53.6)	9 (39.1)	
TNM stage			
I	10 (8)	1 (4.3)	0.007
II	49 (38.5)	3 (13)	
III	56 (44)	19 (82.6)	
IV	12 (9.5)	0 (0)	
Histology			
Non-Mucinous carcinoma	107 (84.2)	10 (43.5)	<0.001
Mucinous carcinoma	20 (15.8)	13 (56.5)	

Figure 2: Kaplan-Meier curves comparing the survival of patients with MSI-H and MSS/MSI-L satus.



We investigated expression analysis of the MMR proteins hMLH1 and MSH2 in the colorectal carcinomas cohort (figure 3). Of 75 MSS and MSI-L cancers investigated, 63 (84%) expressed hMLH1 (figure 4). Of the MSI-H cancers, 12/23 (52%) expressed hMLH1 ($p<0.05$). Of the MSI-H cases, all cases do not expressed the MSH2 protein (figure 5).

Figure 3: Immuhistochemical expression of MLH1 and MSH2. A : healthy mucosa (MLH1 X400) ; B : positivity of MLH1 in the tumor (MLH1X250), absence of MLH1 inset of figure (MLH1X200) ; C : healthy mucosa (MSH2 X100) ; D : positivity of MSH2 in the tumor (MSH2X250), absence of MSH2 inset of figure (MSH2X200).

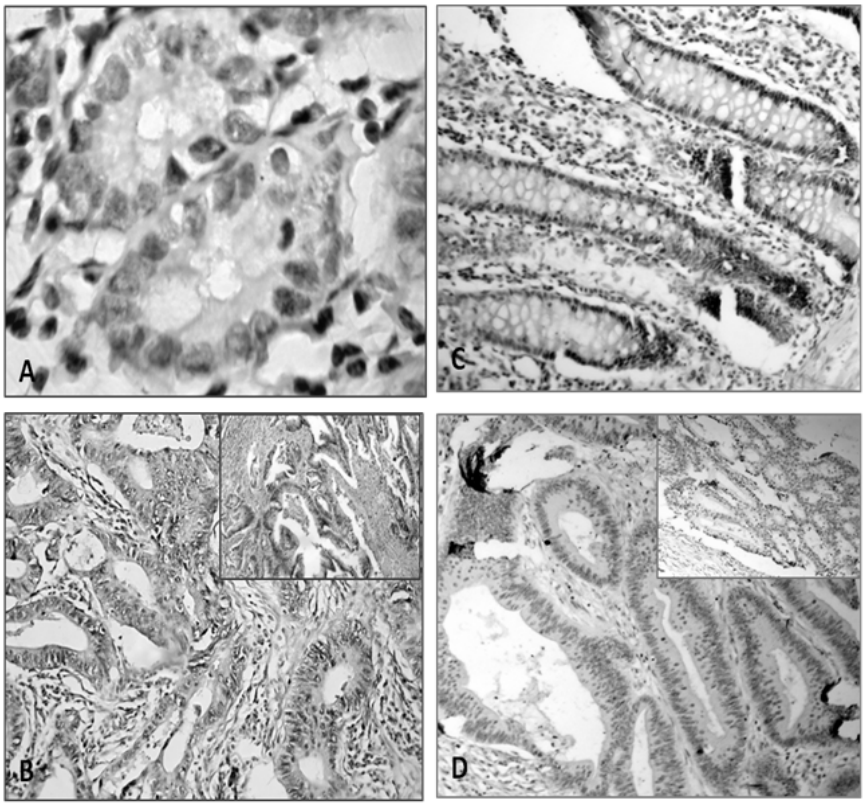


Figure 4: Immuhistochemical expression of MLH1 and MSH2 for the MSS phenotype. Right MLH1 (MLH1 X200) and left MSH2 (MSH2 X 200).

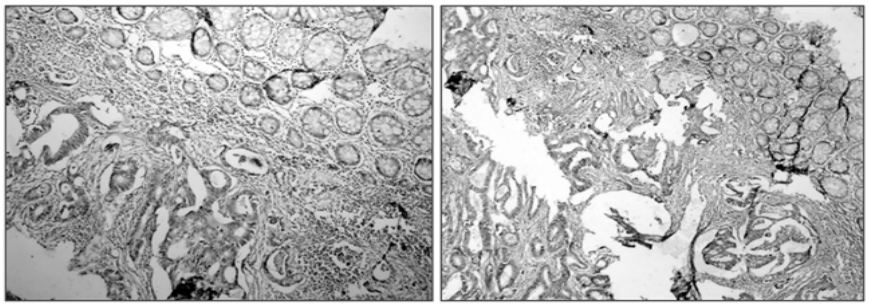
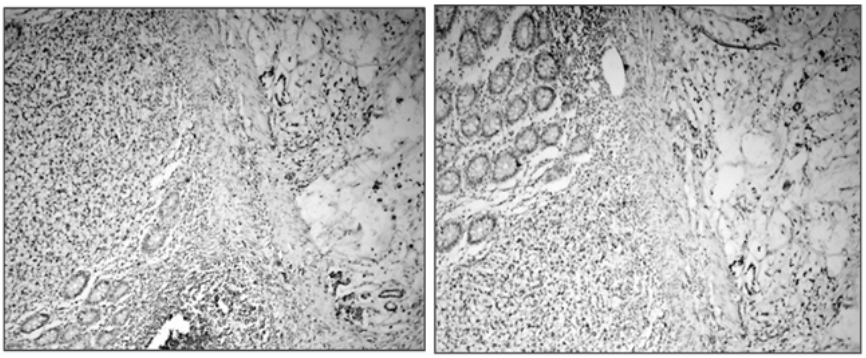


Figure 5: Immuhistochemical expression of MLH1 and MSH2 for the MSI-H phenotype. Right MLH1 (MLH1 X200) and left MSH2 (MSH2 X 200).



Subsequently, we performed the methylation status of the hMLH1 promoter. We are able to successfully perform the MSP assay in 23 MSI-H, 42 MSI-L and 33 MSS tumors (figure 6). As expected, most of the MSS/MSI-L (59/75) cancers were unmethylated at the hMLH1 promoter, while 6 of 23 MSI-H cancers were unmethylated (p<0.001) (table 3, figure 7A).

Figure 6 : Methylation-specific PCR analysis of somes cases of colorectal cancinomas, U and M indicates the unmethylated and methylated fragments, respectively. MP, molecular weight.

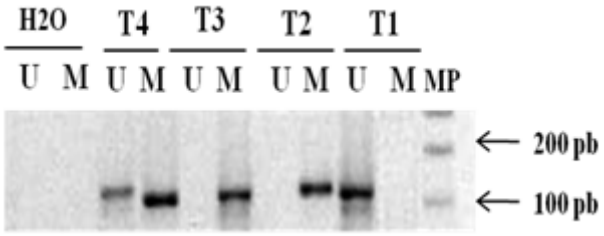
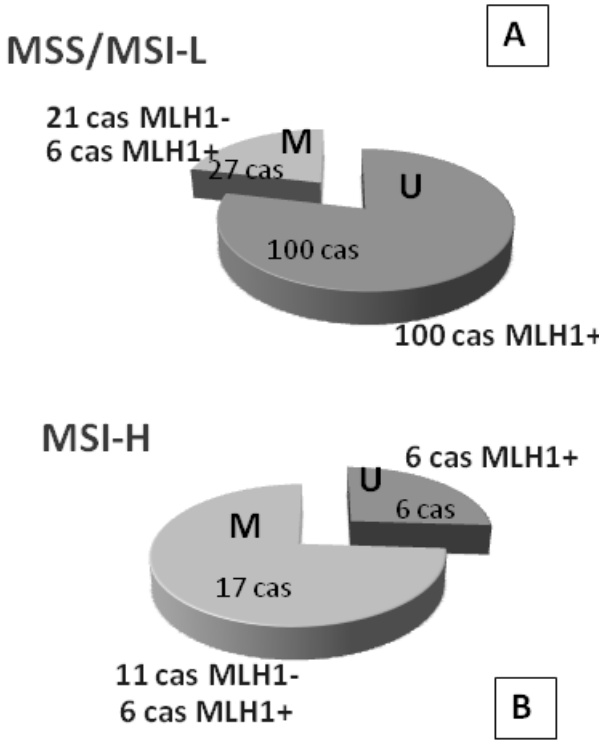


Figure 7 : Methylation status of hMLH1 promoter and immunohistochemical expression of MLH1 in A: MSS/MSI-L cases and B: MSI-H cases.



We further correlated hMLH1 expression with the hMLH1 promoter methylation status of the MSI-H tumors. Of the methylated MSI-H cases, 6/17 (35%) expressed hMLH1 protein while 6/6 (100%) of the unmethylated MSI-H were positive for hMLH1 staining. Of 11 MSI-H cancers with loss of hMLH1 expression, all cases (100%) were also methylated while 50% MSI-H cancers (6/12) with positive immunostaining for hMLH1 were methylated at the hMLH1 promoter (p<0.001) (table 3, figure 7B). We thus were able to demonstrate the close relationship of hMLH1 protein expression with the hMLH1 promoter methylation status.

Table 3 : Methylation status according to the degree of MSI

	MSS/MSI-L (%) n= 75	MSI-H (%) n= 23	P value
Unmethylated	59 (78.7)	6 (26)	<0.001
Methylated	16 (21.3)	17 (74)	

DISCUSSION

In 10–20% of patients with colorectal cancer, carcinogenesis is due to genomic defects in the mismatch repair machinery. Defective DNA repair as a result of germ-line mutations has been linked to sporadic colorectal carcinoma, and also to those carcinomas arising in HNPCC syndrome. In both settings, the mutations and promoter hypermethylation occur mainly in the genes hMLH1 and hMSH2 of the mismatch repair system, and result in loss of their expression [18]. Further, defects in the mismatch repair process with subsequent base pair mismatches lead to MSI [13, 18]. Since the failure of the repair system as a cause of genomic instability is associated with a better prognosis [18, 19] many different microsatellite loci have been used to identify MSI in tumours for diagnostic and prognostic purposes [13].

Molecular classification of colorectal cancer based on its molecular features has important implications regarding prognosis and might influence future treatment strategies. It has been shown that MSI-H sporadic colon cancers have in vitro resistance to commonly used chemotherapeutic drugs compared to cancers with the MSS and MSI-L phenotype [20]. The MSI-H phenotype arise through the defects of the human MMR system, notably through transcriptional silicing of the hMLH1 gene by aberrant methylation of its promoter [12]. Promoter methylation has been demonstrated to inhibit hMLH1 protein expression [21]. So far, MSI-H cancers have been diagnosed by MSI testing using a panel of consensus MSI markers [11]. By definition, tumors with band shifts in 30-40% or more of the tested MSI markers are scored as MSI-H.

MSI-H can be distinguished from MSI-L cancers by a set of clinical and pathological features that includes female predominance, proximal location, mucinous histology, poor differentiation, and the demonstration of mismatch repair gene deficiency, most of which have diagnostic utility [22]. In this study, MSI-H tumours occurred more frequently in the proximal colon, tended to have a mucinous predominance and often exhibited a stage III of TNM. Regarding this last point, in several other studies early-stage disease has been associated with the MSI phenotype [23-25], but we did not find this association in this Tunisian study.

In our study, kaplan–Meier survival curves and log-rank analysis showed that MSI-H was associated with better prognosis than MSS/MSI-L, although no significant difference was found in this study (p=0,27). Most of data from different studies in colorectal and gastric cancer are consistent in defining a better outcome for patients with a MSI-H phenotype [26-29].

With regard to the correlation of hMLH1 protein expression and hMLH1 promotor methylation status, several study was demonstrated that MSI-H phenotype arises through the defects of the human MMR system, notably through transcriptional silicing of the hMLH1 gene by aberrant methylation of its promotor. Promotor methylation has been demonstrated to inhibit hMLH1 protein expression [12, 13, 17, 21, 27]. In our study, we intended to correlate MSI, hMLH1 expression and hMLH1 promoter hypermethylation in a cohort of Tunisian sporadic colorectal carcinoma patients. First, we demonstrated the expected frequency of the MSI phenotype in sporadic colorectal cancer with 15% of all cancers being MSI-H. Interestingly, only 48% of these cancers showed absence of hMLH1 expression. However, 84% (63/75) of MSS/MSI-L cancers did not express hMLH1 protein. Others study showed a specificity of 100% of immunohistochemistry in correctly diagnosing the MSS phenotype in cancers with positive hMLH1 and hMSH2 expression [30-34].

The fact that MSI is evident in colorectal carcinoma and that MSI-H and aberrant promorter methylation of hMLH1 are observed simultaneously, suggests that MSI-H and hypermethylation are dependent on each other. It has been convincingly shown that the MSI-H phenotype is correlated in most cases with aberrant methylation of the hMLH1 promotor which influences the transcriptional activity of the gene [12, 35, 36]. We therefor investigated the promorter methylation status of our entire tumor cohort with the MSI status. We found that most MSS/MSI-L cancers had an unmethylated and, presumably, transcriptionally active hMLH1 promoter. However 21.3% of the MSS and MSI-L cancers showed methylation. For the MSI-H phenotype, 74% of cases demonstrated promoter hypermethylation. Correlating the immunohistochemistry finding and the promoter methylation status in MSI-H cancers, we found an association between negative immunohistochemistry and promoter methylation, as 100% of the MSI-H expressing hMLH1 were unmethylated and 65% of the MSI-H lacking hMLH-1 were methylated.

About the cases that showed hMLH1 expression but the methylated status of hMLH1 promoter, it would have been interesting to obtain data on DNA sequencing on those cases in order to investigate if those tumors harbor somatic missense mutations that inactivate protein function.

As it has been shown before, that among 10% to 15% of the patients with colorectal cancer who have MSI-H, approximatively 70% to 80% exhibit epigenetic gene silencing of the mismatch repair gene, hMLH1 [35, 36]. These features are similar to our Tunisian study and those previously described in colorectal cancers.

In summary, our study of a set of Tunisian population showed that MSI-H phenotype was right-side and exhibit stade III of TNM. Furthermore, the relative correlation of hMLH1 expression and promotor hypermethylation for the MSI is similar to that reported for other studies.

In terms of clinical course and after review of the literature, the predictive effectiveness of chemotherapy by Fluorouracil (5-FU) for patients with MSI-H genotype remains controversial. Elsalah et al., reported that the MSI status was predictive of good response to 5-FU in the adjuvant prescribed [37]. Moreover, the results of a retrospective study by Ribic et al., on 570 cases of CRC stage II and III treated with 5-FU, suggest that five year survival of patients with MSI-H status was significantly higher than the MSS patients in the absence of adjuvant chemotherapy. But in the presence of chemotherapy, the five year survival of MSS patients was higher than the MSI-H patients. The traitment seemed to benefit as well as patients with MSS genotype, leading the authors to conclude that chemotherapy was not indicated for the MSI-H patient in stage II /III after resection of tumor [38].

In this context, the determination of MSI status of the patient is essential and would guide the clinician for the treatment to follow.

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