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Original Article

Clinicopathologic characteristics of colorectal cancer with microsatellite instability

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ABSTRACT

Colorectal cancer (CRC) can be classified according to the level of microsatellite instability (MSI) exhibited by the tumor. The aim of this study was to determine MSI status in CRC from Tunisia and to identify clinical and pathological characteristics of MSI-H tumors.

Microsatellite status was determined by polymerase chain reaction amplification using standard markers (BAT25, BAT26, D2S123, D5S346 and D17S250, the Bethesda panel) in 44 CRC cases. Molecular results were correlated with pathological and clinical features.

Six CRC cases (13.8%) showed high-level instability (MSI-H), 14 cases had low level instability (MSI-L), and the remainders were stable (MSS). Immunohistochemical analysis showed loss of MSH2 protein in 3 cases among the 6 MSI-H tumors, whereas no silencing of MLH1 or MSH6 was found in any case. Significant differences in age and family history of cancers were observed between MSI-H and MSS/MSI-L groups ($p = 0.01$ and $p = 0.002$). However, statistical analysis showed that there were no significant differences between MSI-H and MSS/MSI-L tumors in terms of tumor location, lymph node involvement and stage of disease. Regarding histological features, MSI-H tumors were more likely to be poorly differentiated ($p = 0.003$), to have a medullary pattern ($p = 0.005$), and to harbor increased numbers of peritumoral lymphocytes ($p = 0.001$).

These findings indicate that careful observation of the tumor morphology can assist in the identification of unstable colorectal cancers requiring molecular investigations.

Introduction

Colorectal cancers (CRC) are considered to belong to the most common cancers in industrialized countries. They are the third most common cancer in men after lung and prostate cancers and come second in women after breast cancer. In Tunisia, CRC have an annual incidence of 10.3/100,000 individuals. However, a significant proportion (9%) of these cancers occurs before age 40 years, suggesting genetic susceptibility [13].

The deficiency of the DNA mismatch repair (MMR) system is a major mechanism for carcinogenesis. It is involved in the development of hereditary non-polyposis colorectal cancers (HNPCC, also called Lynch syndrome) and in nearly 20% of sporadic CRC [3]. This deficiency is responsible for microsatellite instability (MSI), resulting from the accumulation of small insertions or deletions that frequently arise during replication of these short repeated sequences [25].

Dysfunction of MMR proteins may be related either to an alteration of a gene at the constitutional level, which is the case with Lynch syndrome mutations constitutional MMR genes (hMLH1, hMSH2, hPMS1, hPMS2 and hMSH6), or to altered gene in somatic tumor. In the latter case, it is often a loss of function of hMLH1 most often involved is its epigenetic repression by methylation of its promoter [34].

Determining the MSI phenotype by molecular biology techniques has been standardized by the international conference organized by the National Institute of Health in 1998 [3], which recommends the genotyping of five markers: three dinucleotide repeat markers (D2S123, D5S346, D17S250) and two mononucleotide repeat markers (BAT25 and BAT26) [3]. The realization of this phenotype, which compares the constitutional and tumor genome of a patient, assumes control of the histological quality of the tumor sample.

Besides a fundamental interest because of the original transformation mechanism, the study of tumors with MSI instability is of great clinical interest. It has indeed been shown in several studies that tumors with MSI had several epidemiological, anatomical, histological and prognostic features [1,14,39].

The aim of the present study was to analyze in a series of CRC from Tunisia the clinico-pathological characteristics of colorectal cancers with microsatellite instability.

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Material and methods

Patients and tumor samples

Patients who had undergone resection of CRC between January and December 2007 were identified from the files of the Department of Pathology, CHU Farhat-Hached of Sousse, Tunisia. Patients with history of familial adenomatous polyposis or preoperative anticancer therapy were excluded from the study. Fifty patients were retained for this study. For all of these cases, matched pairs of colorectal tumor and normal adjacent tissues were obtained as formalin-fixed, paraffin-embedded specimens.

Clinical information regarding gender, age at diagnosis, location of the tumor (classified as proximal or distal in reference to the splenic flexure of colon), tumor extension according to the classification of UICC/AJCC, personal and family history of cancer, was retrieved from available hospital records for every patient.

Review of tissue sections was carried out by two pathologists (MT and SZ) without knowledge of the tumor MSI status. At least three histologic hematoxylin–eosin stain slides were reviewed per case. Several histological parameters were evaluated semiquantitatively.

Histological tumor type and grade were determined according to the World Health Organization classification [11]. The percentage of mucin was quantified under low power magnification; we divided our patients according to the percentage of this component into three groups: <5%, 5–50%, >50%.

Considered as "medullary-type" cancers, a subgroup showed poor differentiation consisting of nests, trabecular, and sheets of small- to medium-sized cells with scant to abundant eosinophilic cytoplasm, frequent mitotic figures and a distinct stromal population of small lymphocytes [1,11]. We divided our patients according to the percentage of this component into three groups: <5%, 5–50%, >50%.

Patterns of lymphocytic infiltration include the presence of intra-epithelial lymphocytes and peritumoral lymphocytes were determined. Tumor-infiltrating lymphocytes based on the finding in a hematoxylin and eosin-stained section of at least four intraepithelial lymphocytes in one field at high magnification [39]. Peritumoral lymphocytes were defined as the cuff of lymphocytes surrounding the deepest point of the advancing front of the tumor [8].

DNA extraction and β-globin amplification

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissues samples as described previously [20]. Briefly, 2–5 sections (5 μm thick) of each tissue sample were cut using a microtome and were put in a microtube containing 400 μl of a lysis buffer (50 mM Tris–HCl at pH 8.5; 1 mM EDTA; 0.5% Tween 20). Then, a volume of 10 μl of proteinase K, initial concentration of 10 mg/ml, was added to the reaction mixture. After incubation overnight at 56 °C, the tubes were boiled for 7 min to inactivate the enzyme. To separate DNA from the other components, centrifugation at 12,000 rpm for 15 min and at 4 °C was conducted, the supernatant, containing the DNA, was collected and stored at −20 °C until use.

The quality of the extracted DNA was assessed by polymerase chain reaction (PCR) amplifying a 268-base pairs (bp) sequence of the housekeeping gene β-globin using a pair of specific primers (globinS: 5′-CAACTTCATCCACGTTCACC-3′ and Gh20: 5′-GAAGAGCCAAGGACAGGTAC-3′) [27]. Samples of high-quality DNA were submitted to microsatellite instability analysis.

Microsatellites analyses

Microsatellite instability was assessed using the five microsatellite markers recommended by the 1997 National Cancer Institute-sponsored consensus conference in Bethesda [3]: two mononucleotide markers, single nucleotide repeats (BAT25 and BAT26) and three dinucleotide markers, repetitions of a pattern of two nucleotides (D5S346, D2S123 and D17S250). Primer sequences are given in Table 1.

Table 1
Primer sequences and PCR annealing temperature for the five Bethesda markers.

Marker	Primer sequence (5′→3′)	Hybridization temperature
BAT25	TCGCCTCCAAGAATGTAAGT TCTGGATTTTAACTATGGCTC	53 °C
BAT26	TGACTACTTTTGACTTCAGCC AACCATTCAACATTTTTAACC	53 °C
D2S123	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC	53 °C
D5S346	ACTCACTCTAGTGATAAATCGGG AGCAGATAAGACAAGTATTACTAG	58 °C
D17S250	GGAAGAATCAAATAGACAAT GCTGGCCATATATATATTTAAACC	48 °C

Optimal annealing temperatures were determined for each primer pair, and 200 ng of template DNA was amplified by PCR in a final reaction volume of 25 μl containing 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA), PCR buffer 1× (10 mM Tris, pH 8.3, 50 mM KCl), 0.2 mM each deoxynucleotide triphosphate (dNTP), and 2.5 mM $MgCl_2$, and 0.3 mM each primer.

The reaction amplifications were carried out on a PTC200™ thermocycler (MJ Research, Watertown, MA, USA). Thermal cycling was performed using the following conditions: initial denaturation at 93 °C for 5 min, followed by 35 cycles of 1 min at 93 °C, 1 min at the specific annealing temperature (Table 1) and 1 min at 72 °C. The reaction was finished with a 10-min extension at 72 °C.

Finally, PCR products were separated by electrophoresis in polyacrylamide mini gels using Mini-Protean 3 System (Bio-Rad, Marnes-la-Coquette, France), stained by ethidium bromide and visualized under ultra-violet light using the GelDoc2000 System (Bio-Rad).

MSI was identified by the presence of novel bands in the PCR product from malignant colorectal tissue as compared with the pattern from non-malignant tissue of the same patient. Each gel was scored independently by two experienced observers (MT and RBG). If there was discordance in their interpretation, the gels were reviewed to provide a consensus result.

Tumors in which none of the markers showed instability were classified as microsatellite stable (MSS). Those with a single unstable marker were classified as MSI-low (MSI-L). Tumors with two or more unstable loci were classified as MSI-high (MSI-H) [3].

Immunohistochemical staining

Immunohistochemistry was performed for MSI-H cases to assess the expression of the MMR proteins: MLH1, MSH2, and MSH6.

Briefly, four micron-thick sections were obtained from formalin-fixed, paraffin-embedded tissue blocks. The tissue sections were deparaffinized in toluene and rehydrated in graded concentrations of alcohol. Endogenous peroxidase activity was blocked by treating the sections with a blocking solution. For antigen retrieval, the sections were treated while boiling in citrate buffer (pH 6.0) in a microwave oven. Sections were then rinsed with Tris-buffered saline (TBS) (0.05 M Tris–HCl, 1.15 M NaCl, pH 7.6) and incubated overnight at 4 °C with primary antibodies against MLH1 (Diagnostic BioSystems, clone: G168-15, dilution of 1:100), MSH2 (Diagnostic BioSystems, clone 25D12, dilution of 1:50), MSH6 (Diagnostic BioSystems, clone: 44, dilution of 1:50).

After rinsing in TBS, the described antibodies were detected using the EnVision+ labeled polymer Kit (DakoCytomation) following the manufacturer's instructions. To visualize immunoreactivity,