Original Article

Microsatellite instability and protein expression of MLH1 and MSH2 genes in young Mexican patients less than 50 years of age diagnosed with colorectal cancer

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Abstract: Diagnosis of colorectal cancer in patients under 45 years old should alert us to possible hereditary forms of this neoplasia. Most cases of hereditary colorectal cancer correspond to Lynch syndrome which is caused by mutations in DNA mismatch repair genes, particularly MLH1 and MSH2. The dysfunction is associated with microsatellite instability which occurs in 95% cases of this syndrome and in 15% of sporadic colorectal cancer. In sporadic colon tumors, downregulation of MLH1 is observed in cases with the BRAF V600E variant, which induces hypermethylation of the MLH1 promoter. Mutation screening for hereditary cancer has impacted the diagnosis, genetic counseling, and early tumor detection in families affected by hereditary colorectal cancer syndromes but mutation screening technologies are seldom available in public health care centers in developing countries. This study aimed to describe immunohistochemistry and microsatellite instability abnormalities in tumor samples archived in a public hospital in Mexico. Paraffin-embedded samples of patients with colorectal cancer, diagnosed at under 50 years old, were studied to analyze correlations among clinical variables, MLH1 and MSH2 protein expression (immunohistochemistry), microsatellite instability (fluorescent PCR-based assay), and BRAF V600E variant (real time PCR). Forty-seven tumor specimens from patients with TNM stage II and above were analyzed. Tumors were mainly located in the proximal colon segment and displayed histologic intestinal variety and infiltration to serosa. Twenty samples showed decreased expression of mismatch repair proteins and 10 of these presented microsatellite instability (7 high and 3 low instability patterns, respectively). There were no instances of BRAF V600E mutation found. Altered MLH1 or MSH2 expression was found in 42.5% of the samples and microsatellite instability was observed in 21.3% of the tumors. These results suggested that about a fifth of the patients were candidates for family assessment and genetic counseling.

Keywords: Colorectal cancer, microsatellite instability, DNA mismatch repair

Introduction

Colorectal cancer (CRC) ranks third in frequency among cancers worldwide. In Mexico, this neoplasm represents the third highest cause of cancer incidence, affecting 6.8 per 100,000 inhabitants [1, 2]. CRC is classified into different groups based on genomic (microsatellite and chromosomal instability) and epigenetic alterations observed in tumors [3, 4]. Thirty percent of patients report family history; between 6-8% of CRCs correspond to hereditary forms. The most common monogenic form of CRC is the hereditary nonpolyposis colorectal cancer (HNPC) or Lynch syndrome, representing around 5% of all gut tumors [5, 6]. The main features of this syndrome include early age presentation (average age at diagnosis 44-60 years), predominant involvement of the right colon, high incidence of synchronous and metachronous colorectal tumors, and association with extracolorectal neoplasms (adenocar-
IHC and MSI screening for colorectal cancer in young

<table>
<thead>
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<th>Table 1. Clinical pathology features of the samples</th>
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Documentation of mutations in MMR genes implicates DNA sequencing technologies that are costly and of limited availability in some clinical settings. Therefore, immunohistochemistry (IHC) studies of the most commonly affected proteins in Lynch syndrome (MLH1 and MSH2) and MSI detection and quantification techniques are alternative methods for selecting candidate patients for family assessment [11]. These methods have not been fully implemented in public health oncology services in Mexico. The aim of this study was to describe IHC (for MLH1 and MSH2), MSI abnormalities, and the BRAF V600E mutation in a collection of tumor samples from CRC patients under 50 years old (y.o.) that were archived in the University Hospital of Universidad Autonoma de Nuevo Leon in Monterrey, Mexico.

Material and methods

Forty-seven cases of paraffin-embedded tissue from surgical resections of colorectal adenocarcinoma were obtained from the archives of the Department of Pathology, University Hospital of the Universidad Autonoma de Nuevo Leon. Data on age, sex, tumor location and size, histological type, degree of infiltration, TNM stage, number of positive nodes, and the presence of polyps were registered for each case (Table 1). This study was approved by the Ethics Committee of the University Hospital of the Universidad Autonoma de Nuevo Leon (reference number BI13-003). This was a retrospective study. Informed consent requirements were waived for this study.

According to selection criteria, all samples included in our study were from patients less than 50 y.o. having been diagnosed with CRC. The study included IHC for MLH1 and MSH2 proteins and BRAF V600E detection in all samples. MSI study was restricted to those samples showing absence of BRAF V600E mutation and decreased MLH1 and/or MSH2 protein expression.
Immunohistochemistry

Five micron sections of each sample mounted on silanized slides were deparaffinized in xylene and subsequently rehydrated in alcohol solutions at progressively lower concentrations. Antigen retrieval was performed in a pressure cooker for 15 minutes. Slides were manually stained with mouse monoclonal antibodies for MLH1 (1:25) and MSH2 (1:25) (both from Biocare Medical Concord, CA). The primary antibody was incubated for 60 minutes and then incubated with the secondary antibody conjugated to streptavidin-biotin. IHC were developed with diaminobenzidine and counterstained with hematoxylin. The interpretation of the IHC was performed following the recommendations of UKNEQAS (United Kingdom National External Quality Assessment) [14]. Nuclear staining was considered positive and internal controls in the adjacent mucosa and lymphocytes were used as well as histologically normal specimens from the cecal appendix. Non-detection of nuclear staining was interpreted as a loss of expression with any of the markers used, with these being normally expressed in the internal and external control.

Detection of BRAF V600E mutation

Tissue selection was based on microscopic examination, by an expert pathologist, of slides stained with hematoxylin and eosin to estimate the area of tumor tissue and subsequently perform a punch biopsy of this area. It was later collected in 1.5 mL Eppendorf tubes for extraction of genetic material.

DNA extraction

Total phenol-chloroform DNA extraction was performed from the tumor and adjacent healthy tissues (for subsequent MSI analysis) that were previously deparaffinized with xylene and digested with proteinase K. Purity and DNA concentrations were analyzed using the NanoDrop ND-1000 spectrophotometer, obtaining results within the acceptable parameters of DNA quality in all of the specimens. Samples were stored at -20°C for later studies.

Detection of BRAF V600E mutation was performed by real-time PCR. Primers and probes previously validated in a Spanish population were chosen, following the conditions established in this reference [15]. DNA from the HT29 cell line (human tumor line derived from colon adenocarcinoma, which is heterozygous for the BRAF V600E mutation, was used as a positive control. Amplification and fluorescence detection was performed using ABI Prism 7000 Sequence Detection System (Life Technologies).

Analysis of microsatellite instability

The MSI Analysis System, Version 1.2 (Promega Biotech), was used to amplify five mononucleotide markers: BAT-25, BAT-26, NR-21, NR-24, and MONO-27. Two pentanucleotide markers were used to confirm that the tumor sample and normal tissue corresponded to the same patient. Tumor DNA and DNA from healthy tissue adjacent to the tumor were amplified according to the manufacturer’s recommendations. PCR products were analyzed by capillary electrophoresis using an ABI 3100 Genetic Analyzer (Applied Biosystems). Data analysis was performed using GeneScan (Applied Biosystems) software. A size difference between alleles of tumor tissue and healthy tissue in one or more of the 5 markers was defined as MSI. Samples were classified as low-MSI when just one allele presented instability and as high-MSI when two or more alleles presented instability.

Statistical analysis

Pearson’s Chi-square ($\chi^2$) test was selected to evaluate differences between the percent frequencies of each variable. Results with a $p$-value ≤ 0.05 were considered statistically significant. SPSS 15.0 V was used to perform the analyses.

Results

Pathological data description

Of the total samples analyzed, 21 cases were women and 26 were men (female/male ratio: 0.81). Mean ages for women and men were 38 and 36 years, respectively. TNM stage III was more prevalent than other stages in the study group. Tumor variants, in order of decreasing frequency, were intestinal, mucinous, and medullary. This frequency was conserved when samples were stratified by alteration (total n = 47; samples with alterations in IHC, n = 20; samples with MSI, n = 10). Twenty-two tumors were located in the proximal colon. Most of the tumors (n = 37) showed a degree of infiltration to the serosa and no tumor was limited to the
Absence of expression of MLH1 only was detected in 16 samples, 1 sample did not express MSH2 only, and 3 did not display expression of both, MLH1 and MSH2 proteins. No differences were observed for IHC abnormalities regarding tumor location and serosa infiltration (Table 1). Clinical data was retrieved for 15 cases from these samples. These patients were treated with surgery and received at least 6 cycles of chemotherapy with schemes containing 5 fluorouracil. Disease progression was evaluated by computed tomography. Nine cases showed progression-free survival 12 months post-therapy, 3 patients had disease progression during the adjuvant treatment, 2 had disease recurrence before 2 years post-therapy, and 1 case presented stable disease after 12 months post-therapy.

**Analysis of BRAF V600E mutation**

The wild-type BRAF V600 allele was identified in all cases and no instances of the mutation were observed. This finding was confirmed by sequencing the BRAF gene in 5 randomly selected samples using DNA from the HT29 cell line as a positive control.

**Analysis of microsatellite instability**

Although all samples showed abnormal IHC (n = 20/47), MSI was observed only in 10 samples (7 with high-MSI and 3 with low-MSI). The BAT26 marker showed instability in all MSI positive samples (Figure 1).

**Discussion**

In this study, protein expression of MLH1 and MSH2, the BRAF V600E mutation, and MSI were analyzed in 47 tumor paraffin embedded samples from patients with CRC less than 50 y.o. Age criterion alone was not suggestive of alterations in the MMR system in 27 out of 47 analyzed samples (as indicated in the Bethesda guidelines for HNPCC), however, this parameter was a useful condition for HNPCC screening since 20 samples (42.5%) showed MMR abnormalities by IHC, particularly in expression of MLH1. In a previous study, expression of MLH1 and MSH2 proteins by IHC in a group of patients with CRC over 50 years was analyzed and found that only 7 cases presented IHC alterations, 6 cases with normal MLH1, and 1 case with MSH2 alteration [16]. Stigliano et al. found 6/70 CRC cases diagnosed at age ≤ 50 with alteration in IHC and/or MSI and no family history of CRC and/or other malignancies of HNPCC spectrum [17]. We reported 7/47 similar cases. Our data indicates that, in addition to age at cancer diagnosis, patient family history is relevant for considering molecular testing for genetic counseling.

BAT-26 is a highly sensitive marker for tumors with MSI and it is suggested as an ideal marker for identifying tumors with MSI [18, 19]. In this study, BAT-26 showed better performance for detecting MSI than the other microsatellite markers, confirming its high sensitivity for detection of tumors with MMR alterations. It has been reported that MSI tumors show certain clinical features that differentiate them from the rest of colorectal cancers, allowing their grouping as an independent subtype [20, 21]. Several studies suggested that hereditary MSI tumors are more frequent in the right colon and rare in the rectum, coinciding with locations found in this study [8, 22].
Some authors have indicated that tumors with MSI have better prognosis and showed some resistance to 5-fluorouracil [11, 23]. In addition, it has also been reported that MSI is more common in CRC at stage II (~20%) than at stage III (~12%) and even less common in stage IV (~4%) [24, 25]. Our study shows a discrepancy in this regard since most of the patients with altered MMR proteins or MSI were in stage III (60%), compared to patients in stage II (40%).

As reported, IHC for MLH1, MSH2, MSH6, and PMS2 increases the sensitivity for diagnosis of HNPCC, making it similar to the sensitivity of MSI analysis with a concordance above 90% [26, 27]. However, in this study, only 10 of 20 samples with abnormal MMR protein showed MSI. This was probably because this study was limited to expression of MLH1 and MSH2 proteins, based on the assumption that up to 90% of alterations in MMR genes are found in MLH1 and MSH2 genes [28]. This discrepancy may result from not including IHC analyses for additional MMR proteins involved in HNPCC. It is also possible that some dysfunctional germline variants will not result in the absence of protein epitopes in the tumor cells [29, 30].

A low frequency of mutations in tumor suppressor genes, like p53, has been suggested as a factor of less aggressive clinical CRC evolution [31]. In our report, we did not detect expression of TP53 in the 4 samples showing alterations in the MLH1 and MSH2 proteins (three of these cases showed no expression of the two MMR studied proteins and MSI-high), consistent with the lower frequency mutation report. Remaining samples were positive for TP53 expression.

MLH1 under-expression in sporadic tumors results from an epigenetic silencing associated to BRAF V600E mutation. This variant was not detected in the analyzed samples and suggests that these alterations involve germline mutations. From the 4 samples with impaired MSH2 protein in this study, 3 samples shared alteration of MLH1 protein, raising the possibility of promoter methylation of the MSH2 in the remaining MMR altered tumor. Although we did not study this possibility, it has previously been described [32].

Early CRC detection programs based on colonoscopy are scarce in Mexico. Protocols to detect molecular alterations (IHC, MSI, and mutation detection) in hereditary colon cancer are practically nonexistent in the country, unlike the institutional programs existing in Europe and North America aimed at offering genetic counseling for family members. Although Mexican health services cover the IHC analysis, it is optional and is not performed routinely. Currently, there is only one institution in our city that routinely includes MSI analysis for patients in stage II, which is a seldom detected stage.

Conclusion

This work integrated analysis of MLH1/MSH2 protein expression, MSI, and BRAF V600E in tumor samples of young patients with CRC in Mexico. The study showed that 42.5% of the patients were candidates for family assessment and genetic counseling. It suggests that IHC is a convenient alternative for detecting HNPCC candidates in hospitals with limited diagnosis resources and that this technique is also useful for gene sequencing selection for mutation detection studies. This work also marks the importance of providing molecular diagnosis in CRC cases younger than 50 years with defects in MMR protein expression and MSI.

Acknowledgements

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Disclosure of conflict of interest

None.

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