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ORIGINAL ARTICLE

Universal determination of microsatellite instability using BAT26 as a single marker in an Argentine colorectal cancer cohort

María Laura González[1,2] · Natalia Causada-Calo[1,2,3] · Juan Pablo Santino[1,5] · Mev Dominguez-Valentin[4] · Fabiana Alejandra Ferro[1] · Inés Sammartino[1] · Pablo Germán Kalfayan[1] · Maria Alicia Verzura[1] · Tamara Alejandra Piñero[1,7] · Andrea Romina Cajal[1,7] · Walter Pavicic[1] · Carlos Vaccaro[1,6]

Abstract Microsatellite instability (MSI) is a hallmark tool for Lynch syndrome (LS) screening and a prognostic marker for sporadic colorectal cancer (CRC). In regions with limited resources and scarce CRC molecular characterization as South America, the implementation of universal MSI screening is under debate for both its purposes. We sought to estimate the frequency of BAT26 in colorectal adenocarcinomas and to determine associated clinical and histological features. Consecutive patients from a CRC registry were included. BAT26 determination was performed in all cases; if instability was found, immunohistochemistry (IHC) and *BRAF* mutation analyses were done, as appropriate. Differences were assessed by chi-squared or Fisher's exact test, or by *T* test or Mann–Whitney. Multiple logistic regression was used to identify factors independently associated with BAT26-unstable tumors. We included 155 patients; mean age was 65.6 (SD 14.4) and 56.1% were male. The frequency of BAT26-unstable tumors was 22% (95% CI 15.7–29.3). Factors independently associated with BAT26-unstable tumors were right colon localization (OR 3.4, 95% CI 1.3–8.7), histological MSI features (OR 5.1, 95% CI 1.9–13.6) and Amsterdam criteria (OR 23.2, 95% CI 1.9–286.7). IHC was altered in 85.3% BAT26-unstable tumors and 70.6% lacked MLH1 expression; 47.8% of these harbored *BRAF* V600E mutation. We provide evidence to link the frequency of BAT26 to an increased diagnostic yield (up to 1.4-folds) of suspected LS cases in comparison to the revised Bethesda guidelines alone. In regions with limited resources, clinical and histological features associated with BAT26-unstable status could be useful to direct MSI screening in sporadic CRCs and may help guide clinical care and future research.

Keywords Microsatellite instability · BAT26 · Colorectal cancer · Lynch syndrome · Universal screening

✉ María Laura González
marialaura.gonzalez@hospitalitaliano.org.ar

1 Programa de Cáncer Hereditario (ProCanHe), Hospital Italiano de Buenos Aires, Buenos Aires, Argentina

2 Gastroenterology Department, Hospital Italiano de Buenos Aires, 4190 Juan D. Perón St. (C1199ABD), Buenos Aires, Argentina

3 Department of Medicine, Farncombe Family Digestive Research Institute, McMaster University, Hamilton, ON, Canada

4 Department of Tumor Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

5 Pathology Department, Hospital Italiano de Buenos Aires, Buenos Aires, Argentina

6 Surgery Department, Hospital Italiano de Buenos Aires, Buenos Aires, Argentina

7 Instituto Universitario, Hospital Italiano de Buenos Aires, Buenos Aires, Argentina

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related deaths in Argentina [1–3]. In the recent years, there has been a dramatic increase in the burden of CRC in South America. This correlates with a demographic and epidemiological transition in many countries of the region, particularly Chile, Uruguay, Brazil and Argentina [3, 4]. In the most recent report from Globocan (2012), the age-standardized incidence of CRC in Argentina was 19.1 and 29.8 per 100,000 in women and men, respectively [1], reaching similar levels of incidence to those in countries with higher levels of human development index, such as Canada and United States [5]. Despite the significant

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progress made in CRC understanding in many developed countries, studies characterizing CRC molecular characteristics in cases from low- and middle-income countries from Latin America are still lacking.

CRC is the result of a complex interaction between environmental, genetic and inflammatory factors [6, 7]. Well-established prognostic biomarkers include *KRAS, NRAS, BRAF* somatic mutations, microsatellite instability (MSI), and CpG island methylation [8]. MSI or immunohistochemical (IHC) testing are strategies to select patients for a subsequent germline diagnostic testing in mismatch repair (MMR) genes. Interestingly, individuals who have *BRAF* mutation and MSI-high (MSI-H) have a better overall prognosis compared to those who have the *BRAF* mutation and microsatellite stable (MSS) disease. Thus, MSI has clinical importance in sporadic and hereditary CRC and has an emerging potential predictive value of response to immunotherapy [9–11].

LS occurs in 3% of all CRCs and is the most frequent cause of hereditary CRC. LS is caused by germline pathogenic variants in one of the MMR genes: *MLH1, MSH2, MSH6* and *PMS2* or deletion in the *EPCAM* gene, which leads to methylation of the adjacent *MSH2* promoter [12, 13]. LS is clinically classified according to the Amsterdam criteria (AC) and/or the revised Bethesda guidelines (BG), both of which include clinical information and family history. The BG also take into account the MSI tumor markers [14–17]. MSI analysis is one of the first approaches for selecting patients for subsequent LS genetic testing with approximately 93% sensitivity [8]. The highest specificity and sensitivity is achieved by the use of the pentaplex PCR panel comprising five quasi-monomorphic mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-22, and NR-24) [18, 19]. BAT26, like all the other repeats, is a segment of non-coding DNA that in tumors with MMR deficiency its length varies in comparison to the somatic DNA. The quasi-monomorphic term is used as the difference in interallelic size is very small for each single locus in the Caucasian population. It has been previously described that BAT26 is a highly sensitive MSI marker, representing an efficient strategy for defining MMR status obviating the characterization of numerous microsatellite markers [20–23]. In line with the low budgets for integrating genetics into clinical practice in South America, BAT26 may represent a low-cost strategy and a risk assessment tool marker.

The primary aim of this study was to estimate the frequency of surgical colorectal adenocarcinomas with MSI determined by BAT26 and to describe clinical, histological and molecular characteristics associated with these tumors. Our overall aim is to improve the molecular and epidemiological characterization of CRC in our region.

Patients and methods

We performed a prospective cohort study based on an Institutional, national certified Colorectal Cancer Registry (REM, Clinicaltrials.gov: NCT02781337), which was established in 2011. At present, the Registry is integrated by 1020 incident CRC surgical patients. It contains comprehensive data including patients' and families' characteristics that is complemented with tissue banks. The present report included patients that entered the Registry between May 2012 and November 2013.

All surgical patients with a confirmed adenocarcinoma in the pathology report during the study period were invited to participate. Patients with personal history of inflammatory bowel disease, diagnosis of any polyposis syndrome, unresectable tumors at diagnosis and types of CRC other than adenocarcinoma (carcinoids, GIST, leiomyosarcomas, Kaposi, melanoma and lymphomas) were excluded. The variables collected were gender, age at diagnosis, family history of CRC, tumor site, type of adenocarcinoma, tumor stage -according to the American Joint Committee on Cancer Staging- and histological features—(Crohn-like features, tumor-infiltrating lymphocytes, medullary growth and undifferentiated tumors). The patients were classified according to the AC and/or Bethesda guidelines into three clinical groups: sporadic, familial or suspected LS. All the procedures were reviewed and approved by the Ethics Committee and Institutional Review Board. Informed consent was obtained from all individuals participating in this study. The present study is reported following the STROBE guidelines.

Study outcomes

BAT26 analysis

DNA was extracted from 5 µm-thick paraffin-embedded tissue sections from both tumor and normal colon mucosa using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA). Microsatellite sequence at BAT26 was amplified using specific primers (BAT26 Forward 5′-AAC CAT TCA ACA TTA ACC C-3′ and BAT26 Reverse 5′-TGA CTA CTT TTG ACT TCA GCC-3′). The PCR conditions were: initial denaturation at 95 °C for period of 3 min, followed by a cycle of denaturation at 95 °C for 45 s, annealing 54 °C for 45 s and in the cycle extension at 72 °C for 1 min, completing the 35 cycles, with final extension at 72 °C for 10 min. Subsequently, the PCR products were loaded onto a 6% denaturing polyacrylamide 8M urea gel stained with silver nitrate 0.2%. Shifts of 3 or more bp in BAT26 were considered BAT26-unstable tumors, an equivalent of MSI, and warranted IHC analysis.

MMR-immunohistochemistry

IHC staining for MLH1, MSH2, MSH6 and PMS2 was performed in BAT26-unstable tumors. The antibodies applied were MLH1 (M1) Mouse Monoclonal Primary Antibody (790-4535), MSH6 (44) Mouse Monoclonal Primary (790-4455), MSH2 (G219-1129) Monoclonal PrimaryAntibody (760-4265), PMS2 (EPR3947) Monoclonal Primary (760-4531) (RocheVentana) using an automated immunostainer (Ventana BenchMark XT, Ventana Medical Systems, Tucson, AZ) and detection system OptiView DAB IHC Detection Kit (Ventana Medical Systems) according to manufacturer recommendations. The slides were evaluated by one pathologist (JPS). Normal colonic crypt epithelium adjacent to the CRC and lymphoid/stromal cells served as internal positive controls for staining. Tumor MMR protein expression was assessed as retained (presence of nuclear staining) or lost (i.e. loss of nuclear staining with retained staining in stromal, inflammatory, or non-neoplastic epithelial cells).

BRAF V600E mutation analysis

BRAF V600E mutation analysis was carried out in the genomic DNA extracted from microdissected tumor tissue. A standard PCR and Sanger sequencing approach was used. Briefly, following successful amplification, by using primers covering codon 600 within exon 15 of BRAF, resulting amplicon was purified and sequenced using the BigDye® Terminator Cycle Sequencing Kit (Applied Bio-systems, Carlsbad, Calif.). Products were analyzed using an ABI3730 Genetic Analyzer (Applied Biosystems). The status of BRAF mutation was verified by sequencing in both directions and all sequences were analyzed by comparison to the wild type sequence.

Universal screening approach

The overall algorithm is described in Fig. 1. As expected, the step 1 in ‘‘biochemical diagnosis’’ of CRC is tumor testing by MSI analysis using BAT26 marker in all tumors. The step 2 involved the MMR-IHC analysis in BAT26-unstable tumors. In the step 3, *BRAF* V600E was conducted when lack of MLH1 expression was found in the IHC analysis. In the other branch, when the BAT26 was stable, clinical guided criteria (AC) were applied to decide whether to proceed with further genetic testing and counseling.

Statistical analysis

Categorical data are presented as percentages with 95% confidence intervals (CI) and continuous data as medians with interquartile ranges (IQRs) or mean and standard deviation (SD), according to distribution. Patients' characteristics were compared using *t* test or Mann–Whitney for continuous data and chi-squared or Fisher exact tests, as appropriate. Univariate relationships between BAT26-unstable tumors and

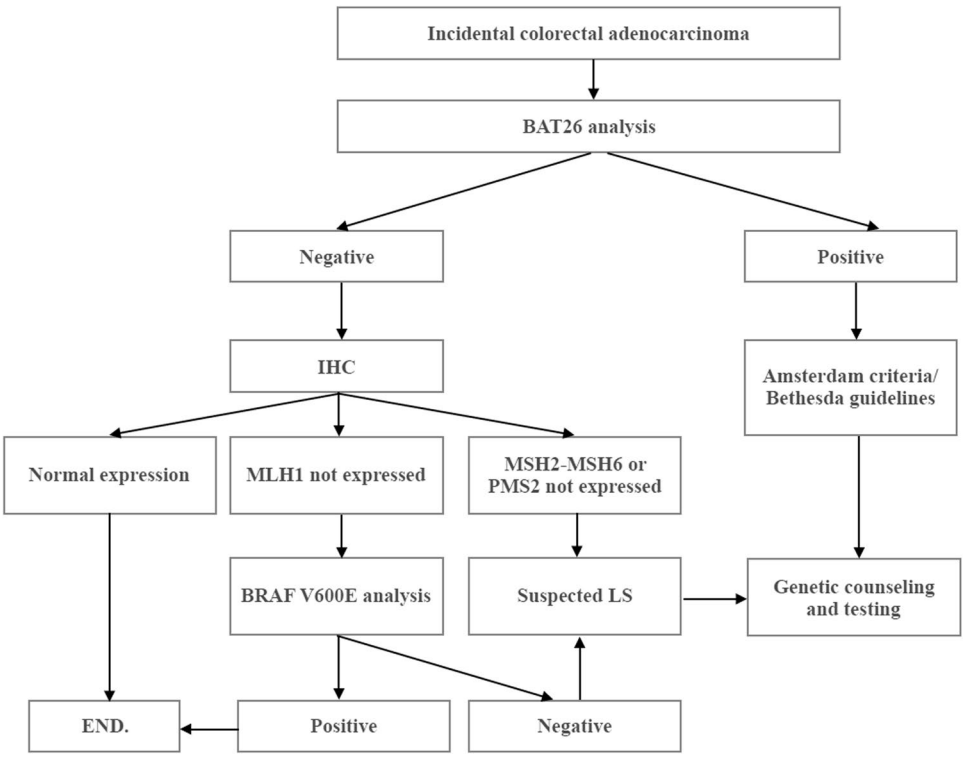

Fig. 1 Testing algorithm

clinical characteristics were evaluated using logistic regression (odds ratios, OR). Multivariate logistic regression analysis was conducted to adjust for potential confounders.

Statistical analysis was performed using IBM SPSS Statistics version 20 software. We defined a *P*-value of < 0.05 for statistical significance and all the tests were two sided.

Results

Study population

The study population was predominantly from Buenos Aires, which is the largest city in Argentina. Three-hundred thirty-eight patients were identified to have CRC diagnosis and underwent colorectal surgery between May 2012 and November 2013. Fifty-three patients fulfilled the exclusion criteria (n = 2 had familial adenomatous polyposis and n = 31 had non-adenocarcinoma CRC) and 20 patients were further excluded because of incomplete demographic data. In total, 285 individuals were eligible to participate in the study. Of them, 232 (81.4%) were invited to participate and 222 (95.6%) agreed. Adequate biological samples were available for 155 patients (69.8%) (Fig. 2).

Clinical and histological characteristics

The mean age at CRC diagnosis was 65.6 years (14.4 SD) and 56.1% were male (Table 1). According to the BG, 112 (72.3%), 11 (7.1%) and 32 (18.7%) patients were classified as sporadic, familial and suspected LS CRCs, respectively. If we apply the AC, sporadic, familial and suspected LS cases frequencies are distributed as follows: 139 (89.7%), 11 (7.1%) and 5 (3.2%).

The tumors were fairly evenly distributed throughout the colon; 34.8% were located in the right colon, 30.3% in the left colon, and 35.5% in the rectum; if the latter two are combined, they represent the majority of the cases (68.5%). Patients displayed mainly stage II and III CRCs (33.5 and 33.5%, respectively). At least one histological features of MSI was found in 33 patients (21.3%).

Characteristics of BAT26 tumors

Thirty-four (22%, 95% CI 15.7–29.3) BAT26-unstable tumors were identified. In the univariate analysis, BAT26-unstable tumors were significantly associated with female sex (P = 0.005), right-side tumor location (P < 0.001), TILs (P < 0.001), poor histological differentiation (P = 0.03), the display of at least one MSI histological feature ($P < 0.001$) (Table 1) and the presence of AC (P = 0.008). In the multivariate analysis, the characteristics that were independently associated with BAT26-unstable tumors were right side location [Odds ratio (OR) 3.4, 95% CI 1.3–8.7]; presence of at least one histological feature of MSI (OR 5.1, 95% CI 1.9–13.6) and from a clinical perspective, the presence of AC (OR 23.2, 95% CI 1.9–286.7) (Table 2).

Amongst patients classified as having sporadic tumors by the use of the BG, 16/24 (66.7%) lacked the expression of MLH1 and PMS2; 2/26 (8.3%) lacked PMS2 alone; 1/26

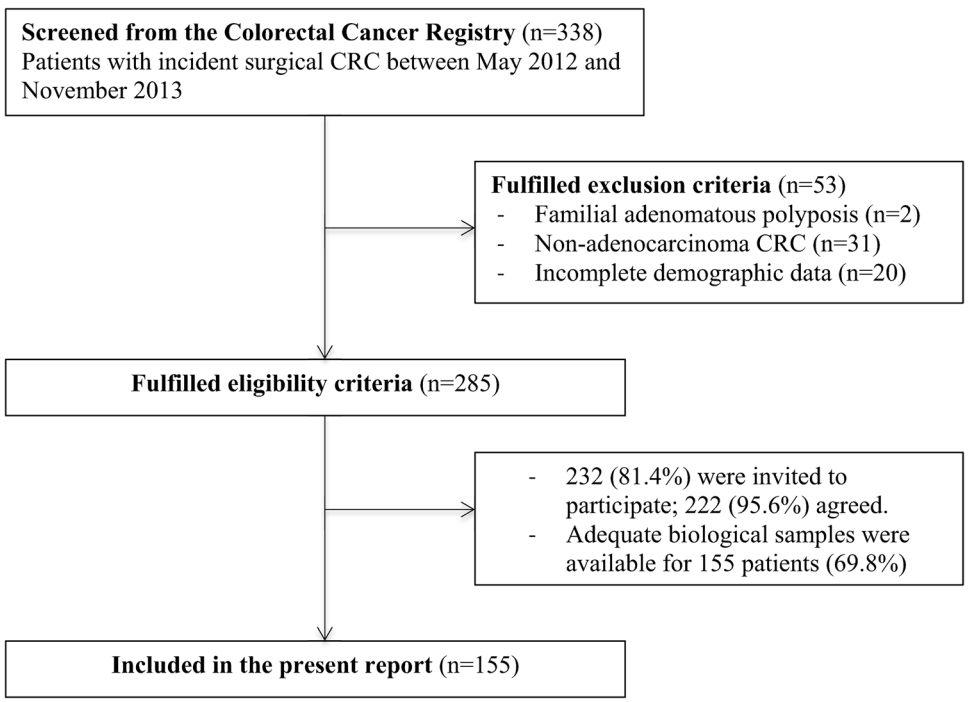

Fig. 2 Selection of study population

Table 1 Characteristics of patients according to BAT26 status

Characteristics	Overall cohort (n = 155)	Patients with BAT26-unstable tumors (n = 34)	Patients with BAT26-stable tumors (n = 121)	*P*-value
Age (years; mean, SD)	65.6 (14.4)	69.2 (15.5)	64.6 (13.9)	0.10
Gender (n, %)				0.005
Female	68 (43.9)	22 (64.7)	46 (38)	
Male	87 (56.1)	12 (35.3)	75 (62)	
Tumor site (n, %)				< 0.001
Right colon	54 (34.8)	22 (64.7)	32 (26.4)	
Left colon or Rectum	101 (65.8)	12 (35.3)	89 (73.6)	
Clinical classification* (%)				
Sporadic	112 (72.3)	24 (70.6)	88 (72.7)	0.8
Familial	11 (7.1)	4 (11.8)	7 (5.8)	0.26
Suspected LS cases	32 (28.1)	6 (17.6)	23 (19)	0.63
Histological features of MSI (n, %)				
Crohn-like	15 (9.7)	6 (17.6)	9 (7.4)	0.09
Signet ring cells	7 (4.5)	3 (8.8)	4 (3.3)	0.16
TILs	17 (11)	14 (41.2)	3 (2.5)	< 0.001
Poor differentiation	12 (7.7)	6 (17.6)	6 (5)	0.03
At least one feature	33 (21.3)	18 (52.9)	15 (12.4)	< 0.001
Tumor staging[§] (n, %)				
I	32 (20.6)	6 (17.6)	26 (21.5)	0.81
II	52 (33.5)	16 (47.1)	36 (29.8)	0.07
III	52 (33.5)	10 (29.4)	42 (34.7)	0.70
IV	19 (12.4)	2 (5.9)	17 (14)	0.25

TIL tumor lymphocytes, *MSI* microsatellite instability

*According to the Revised Bethesda Guidelines

[§]According to the American Joint Committee on Cancer Staging

Table 2 IHC pattern of BAT26-unstable tumors according to the clinical classification using the revised Bethesda guidelines

Immunohistochemistry pattern	All BAT26 unstable tumors (n = 34)	Sporadic (n = 24)	Familial (n = 4)	Suspected LS cases* (n = 6)
MLH1-PMS2 absence (n, %)	24 (70.6)	16 (66.7)	3 (75)	5 (83)
PMS2 absence alone (n, %)	3 (8.8)	2 (8.3)	0	1 (17)
MSH2-MSH6 absence (n, %)	2 (5.9)	1 (4.2)	1 (25)	0
Normal expression (n, %)	5 (14.7)	5 (20.8)	0	0

*According to the revised Bethesda guidelines

(4.2%) MSH2 and MSH6 and the remaining 5/24 (20.8%) had normal IHC protein expression. Of those classified as familial cases, 2/4 (50%) lacked the expression of MLH1 and PMS2, 1/4 (25%) of MSH2-MSH6 and 1/4 (25%) of PMS2 alone. Lastly, in those fulfilling criteria for suspected LS cases, 5/6 (83) lacked MLH1-PMS2 expression and 1 (17) of PMS2 alone (Table 3).

BRAF V600E mutation analysis

Overall, from CRC sporadic cases, 24/112 (21.4%) were BAT26-unstable and from these, 16/24 (66.7%) were MLH1-negative. *BRAF* V600E mutation was found in 11/16

Table 3 Factors independently associated with BAT26-unstable tumors

Characteristic	Odds ratio (95% CI)*	*P*-value
Right-colon tumor site	3.4 (1.3–8.7)	0.01
Histological features of MSI	5.1 (1.9–13.6)	0.001
Amsterdam criteria	23.2 (1.9–286.7)	0.01

MSI microsatellite instability

*Multivariate logistic regression analysis

(68.7%) of the patients. Therefore, the percentage of sporadic patients with the BRAF *V600E* mutation was 9.2% (11/112). Amongst the patients with familial criteria, 4/11 cases were BAT26-unstable and 2/4 (50%) were MLH1-negative tumors; the mutation was not found in neither. In the cases of suspected LS by the BG, the IHC analysis showed lack of MLH1-PMS2 expression in 5/6 (83%) of BAT26-unstable cases, *BRAF* V600E analysis was done in one patient and not found. In the remaining, *BRAF* V600E was not investigated due to inadequate biological samples. However, these patients fulfilled both BG and AC, and therefore needed to undergo genetic testing.

Detection yield of suspected LS cases: universal screening versus clinically-targeted strategies

Amongst sporadic cases defined by the BG, the universal screening with BAT26 identified 8/112 (7.1%) patients with an indication to undergo genetic testing to rule out LS. In the group of familial cases, which were those that did not fulfill the BG for suspected LS but had a family history of CRC, the universal screening strategy identified 4/11 (36.4%) patients at risk of MMR germline mutations. Thus, the molecular screening was able to detect 12 additional cases that would not have been studied according to the BG applied alone. Overall, the combination of the molecular screening and the BG detected 44/155 (28.4%) suspected LS cases in comparison to 32/155 (20.6%) identified with the use of BG alone. In summary, the first strategy increased the detection yield of suspected LS cases by 1.4-folds.

Discussion

In the present study, we found that the frequency of BAT26-unstable tumors in unselected surgical patients with colorectal adenocarcinoma was 22%. Factors that were independently associated with BAT26-unstable status were proximal localization of the tumor, AC and the presence of MSI-histological features. Moreover, in comparison to the revised Bethesda Guidelines applied alone, the universal screening strategy with BAT26 as a single marker increased the detection yield of suspected LS cases by 1.4-folds.

MSI determination, both in the context of LS diagnosis and as part of the molecular characterization of sporadic CRC, has important implications for CRC prevention, prognosis, treatment and surveillance [14, 24, 25]. The first formal guidelines for the clinical diagnosis of LS were the AC, which include family history, clinical and histopathological features. It has been suggested that these criteria are neither sensitive nor specific enough for LS screening [26–29]. For this reason, the Bethesda guidelines were developed to aid in the identification of individuals who should be considered at risk and need further evaluation of MSI and germline mutations of the MMR genes. In addition, MSI status plays a key role in sporadic CRC and universal testing is currently recommended [30].

Conversely, a recent report suggests that targeted screening costs 2- to 7.5-fold less compared to universal strategies and rarely misses LS cases [31]. In this sense, in low- and middle-income countries, the advantage of universal screening over a clinical targeted strategy for the detection of LS remains unclear. Furthermore, in South America, data on molecular CRC profiling is scarce, which makes the implementation of foreign guidelines extremely challenging. Indeed, the incorporation of universal screening for MSI in all CRC cases is under debate in our region. However, if the prevalence of MSI CRC is 22% as we describe, our findings may help guide clinical care and future research.

One study from Brazil [32] showed that MSI, determined by the use of BAT26 alone, correlated well with proximal localization of the tumors and poor histological differentiation, which is in concordance with our findings and that of several other reports [15, 33, 34]. On the other hand, they reported a frequency of MSI-CRC of 12%, which is lower compared to our results (22%). Ethnicity background is a potential explanation for this difference. Brazil, in comparison to Argentina, has a higher African ancestry; it has been described that in African populations polymorphisms are common in BAT25 and BAT26, whilst more than 95% of European-descendants do not display any variations [18]. Other explanation may be the methodology, i.e. not inclusion of a normal DNA sample which may underestimate the MSI frequency when using BAT26 alone. However, the frequency of MSI tumors found in our study is comparable to that of other studies that used di- and mononucleotide MSI markers [15, 35]. We thus believe that in our population, BAT26 does not differ significantly from the strategies used in previous studies.

In the IHC analysis, overall we found that the majority of the BAT26-unstable tumors lacked the expression of MLH1 and PMS2. Among clinically sporadic cases (as per BG), this prevalence was 66.7 and 68% of these harbored the *BRAF* V600E mutation. Thus, 45.8% of the patients without family history of CRC or Bethesda criteria were diagnosed as MSI-sporadic cancers. These findings relate to previous data from a study in families fulfilling AC from Latin America in which most of the germline mutations were found in *MLH1* (60%) [36].

Our study has several limitations that need to be acknowledged. First, and most importantly, we did not determine BAT26 performance to diagnose MSI in our population by comparing it with the pentaplex panel or the IHC, due to implementation barriers and costs. Therefore, we were unable to provide important estimates such as BAT26 sensitivity, specificity and predicted values.

However, we based our decision to use BAT26 as many previous studies have pointed out that it can be helpful to screen for MSI; in a report from Zhou et al., BAT26 correctly discriminated the MSI status in 539 of 542 tumors (99.5%) [22]. Indeed, in our study, BAT26 determination was able to increase by 1.4-folds the diagnostic yield of suspected LS cases compared to the BG applied alone. Second,, we did not analyze the genetic mutations of suspected LS cases to know the frequency of LS in this cohort, as it was not a pre-specified objective of the study. Conversely, our study has several strengths. Patients are part of a large, prospective and widely comprehensive national certified CRC registry that encompasses tissue-bank, clinical, molecular, epidemiological and surgical data; the quality of the demographic information is accurate and was prospectively collected in all cases. We made an extensive effort to characterize BAT26 in all patients and, in those who had BAT26-unstable tumors, we performed IHC and *BRAF* V600E mutation analyses, when appropriate. In addition, this is the first report from South America that provides an insight into the molecular characteristics of incidental colorectal adenocarcinoma by the use of BAT26 marker for MSI characterization with subsequent IHC and *BRAF* V600E determinations.

To conclude, the frequency of BAT26-unstable colorectal adenocarcinomas in the Argentinean population appears to be similar to other regions with a high prevalence of CRC. The use of BAT26 as single marker for universal MSI screening in combination with the BG increased the diagnostic field of suspected LS cases compared to BG applied alone. Lastly, specific clinical and histological features that are independently associated with BAT26-unstable status could be useful to identify a subset of patients with sporadic CRCs for targeted MSI screening in areas with limited resources. Future research assessing the cost-effectiveness of universal screening in our region is warranted.

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Author contributions MLG: concept and design, data interpretation, manuscript writing and scientific discussion. NCC: statistical analysis and data interpretation, manuscript writing. JPS: histopathological and IHQ analysis. MDV: contributed to manuscript writing and scientific discussion. FAF and IS: data collection. PGK and MAV: scientific discussion. TAP and ARC: BAT26 and BRAF V600E determination. CV: concept and design, manuscript revision and scientific discussion.

Compliance with ethical standards

Conflict of interest María L. González and Carlos Vaccaro received a grant from the Instituto Nacional del Cáncer, Argentina. The remaining authors declare no conflict of interest.

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