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# CAT25 defines microsatellite instability in colorectal cancer by high-resolution melting PCR

AG Sánchez[a,b], I Juaneda[c], H Eynard[c], AL Basquiera[d], E Palazzo[d], P Calafat[e], V Palla[e], PA Romagnoli[f] and T Alvarellos[a,b]

[a]Laboratorio de Biología Molecular e Histocompatibilidad, Hospital Privado Universitario de Córdoba, Córdoba, Argentina; [b]Instituto Universitario de Ciencias Biomédicas de Córdoba, Córdoba, Argentina; [c]Servicio de Cirugía, Hospital Privado Universitario de Córdoba, Córdoba, Argentina; [d]Servicio de Oncología, Hospital Privado Universitario de Córdoba, Córdoba, Argentina; [e]Servicio de Anatomía Patológica, Hospital Privado Universitario de Córdoba, Córdoba, Argentina; [f]Centro de Investigación en Medicina Traslacional Severo Amuchastegui (CIMETSA), Instituto Universitario de Ciencias Biomédicas de Córdoba, Córdoba, Argentina

**ABSTRACT**

**Background**: CAT25 ($T_{25}$mononucleotide repeat of the Caspase 2 gene), is a promising DNA marker for detecting microsatellite instability (MSI) in colorectal cancer. CAT25 has the potential to be incorporated into the Bethesda panel, a commonly used panel of DNA microsatellites, or replace it in its entirety. We aimed to develop and validate a high-resolution melting-PCR (HRM-PCR) method for CAT25 instability detection in clinical samples.

**Methods**: The instability of CAT25, BAT25 (a poly(A) tract occurring in *c-kit*) and BAT26 (a poly(A) tract localized in *hMSH2*) microsatellites were assessed in DNA from tumour and peripheral blood obtained from 110 patients with colorectal cancer using HRM-PCR and capillary electrophoresis. Immunohistochemistry (IHC) staining for MSH2, MSH6, MLH1, and PMS2 enzymes was performed on tumours with jigj MSI. Allelic size variation of CAT25 was analysed on peripheral blood DNA from 208 healthy volunteers.

**Results**: The HRM-PCR for CAT25 was validated in clinical samples. CAT25 showed a tight range of 64–66 base pairs. Of 110 tumours, 11 had High MSI, later confirmed by IHC. CAT25 defines MSI alone as well as when used together with BAT25 and BAT26. CAT25 results provided 100% predictive values and $p < 0.0001$ to classify a tumour as having high MSI.

**Conclusions**: We developed and validated a new HRM-PCR assay to detect CAT25 instability. Our findings showed a limited allelic size variation of CAT25 and highlighted to CAT25 as a promising marker for MSI analysis.

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CAT25-marker; colorectal cancer; high-resolution melting; microsatellite instability; real-time PCR

## Introduction

Microsatellites are sequences of repetitive DNA units (1 to 7 base pairs), and are likely sites of replication errors due to a failure of the DNA mismatch repair (MMR) system during DNA replication resulting in microsatellite instability (MSI). The presence of MSI is indicated by the different number of base pairs of microsatellites from tumour DNA, with respect to the number of base pairs present in the normal DNA as can be found in peripheral blood cells. MSI screening is a well-established assay for defining Lynch syndrome [1]. Individuals with this condition have a 50–70% lifetime risk of developing colorectal cancer and 40–60% are at risk of developing endometrial cancer and other malignancies [2]. MSI status has also been proposed as a prognostic factor related to survival and is postulated as a predictive factor of response to the adjuvant therapy for patients with colorectal cancer [3,4]. Recent data have shown the importance of MSI testing for the selection of patients for immunotherapy using anti-PD-1/PD-L1 therapy [5].

The Bethesda panel is frequently used to assess the presence of MSI in colorectal cancer. The panel consists of two mononucleotides, BAT25 (a poly(A) tract occurring in *c-kit*) and BAT26 (a poly(A) tract localized in *hMSH2*gene) along with three dinucleotide markers (D17S250, D5S346, and D2S123) [6]. However, as this panel has limitations, only the use of mononucleotide markers has been suggested for MSI testing [7]. Furthermore, the use of mononucleotide microsatellites with limited allelic size variation could potentially avoid testing the corresponding normal DNA present in peripheral blood samples, often used as reference for MSI analysis [8,9]. In line with this, CAT25, a mononucleotide microsatellite with 25 repetitions of thymine located in the 3'untranslated region of the CASP2 gene has assessed for MSI testing [10]. Different groups have demonstrated the usefulness of CAT25 in terms of clinical efficacy, suggesting that it is comparable to all the five microsatellites proposed in the Bethesda panel [10–13]. These highlight the importance of further studies of CAT25. Here, we developed and validated a new HRM-PCR technique for CAT25 instability detection, which is usually performed using capillary electrophoresis. The HRM-PCR technique is simpler and faster

CONTACT AG Sánchez agsanchez78@outlook.com Hospital Privado Universitario de Córdoba, Naciones Unidas 346, Córdoba 5016, Argentina

technique than capillary electrophoresis [14,15]. HRM-PCR works in a closed-system environment, which also reduces contamination risks with amplicons [14–16].

We present data supporting the use of CAT25 MSI testing, using colorectal tumours as a model, both alone and together with BAT25 and BAT26.

## Materials and methods

One hundred and ten individuals (43 women/67 men) with colorectal adenocarcinoma diagnosed by histopathological analysis and treated in our hospital were studied. The mean age was 66 years (range: 30–89). The number and tumour location were 39 in the right colon, 36 in the left colon, 4 in the transverse colon, 11 in rectosigmoid and 20 in the rectum. The cancers were classified according to The International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) guidelines as follows: 19, 33, 34, and 19 tumours recorded as stage I, II, III and IV respectively. Five tumours could not be classified by their stage. At diagnosis, 27 patients presented with metastases and in several of them, a carcinogenic involvement of more than one location was found. The number and anatomical sites affected by metastases were: 21 livers, 4 lungs, 2 bladders, 3 peritonea, 1 omentum, and 1 bone tissue. Before colorectal tumour removal surgery, there were 3 patients with rectal cancer who received neoadjuvant therapy with radiotherapy and chemotherapy (leucovorin and 5-fluorouracil). After colorectal surgery, adjuvant therapy was administered to 51 patients according to medical criteria (leucovorin, 5-fluorouracil, capecitabine, irinotecan, oxaliplatin, bevacizumab using one drug or a combination of them). The average overall survival at 60 months after colorectal surgery was 43 months.

Five mL of peripheral blood and a piece of fresh tumour tissue were obtained from each patient. The CAT25 allelic pattern was determined using genomic DNA from peripheral blood (2.5mL) of 208 (104 women/104 men) healthy volunteers (control group). The study was approved by the Ethics Committee of the Hospital Privado Universitario de Cordoba and a written informed consent was obtained from all individuals.

Genomic DNA from peripheral blood was extracted using MagNA Pure Compact Nucleic Acid Isolation Kit I following manufacturer's instructions (Roche, Mannheim, Germany). This was also used to obtain Genomic DNA from fresh-frozen tumours as previously were performed by Xicola et al. [17] after a proteinase K (Promega, Madison WI, USA) digestion step (2 mg/mL overnight at 56 ºC). Nucleic acid concentration was determined with an UV spectrophotometer (MaestroGen Inc, Hsinchu City, Taiwan).

The novel SmallCAT25 primers (Forward; 5′-CTTCCCA ACTTCCCTGTTCTTT-3′ and Reverse; 5′-GGCGACAGAGCG AGACT-3′) were generated from GenBank accession number NM_032982 using PrimerQuest software (Integrated DNA Technologies, Coralville, USA) [18]. These primers were created to generate a small amplicon (65bp) and to avoid Alu sequences as suggested [13]. The procedure was carried out following criteria for HRM-PCR setting [19]. On HRM-compatible instruments: Eco Real-time PCR system (ECO) (Illumina, San Diego, USA) for technique validation, and Rotor-Gene 6000 (RG) (Qiagen, Hilden, Germany) and LightCycler Z480 (Z480) (Roche, Mannheim, Germany) for the robustness assay (only for CAT25 analysis). HRM-PCR was accomplished in a total reaction volume of 10 µL containing: 0.2 µL of each SmallCAT25 primer (10 pmol/µL), 5 µL of Kapa HRM Fast master mix 2x (Kapa Biosystems, Wilmington, USA) 1.2 µL of $MgCl_2$ 25 mM (Kapa Biosystems, Wilmington, USA), 1 µL of DNA template (25ng/µL) and 2.4 µL of water. The HRM-PCR cycling conditions were: 95 °C for 3 minutes followed by 42 cycles of 95 °C for 5 seconds and 64 °C for 30 seconds. Before HRM step, the products were heated to 95 °C for 10 seconds and cooled to 40 °C for 5 seconds. Data for HRM analysis were collected within the range of 65 to 95 °C, rising at 0.1 °C/second (RG and ECO instruments) or 0.02 °C/second with 25 acquisitions/second (Z480). Procedure time (ECO instrument): 55 minutes.

Validation parameters for CAT25 HRM-PCR analysis were performed according to the guidelines for molecular genetics techniques as previously published [20].

Robustness determination was as follows: for evaluation of the template amount variation, DNA of Tumour 109/peripheral blood 109 (from the same patient) were added in triplicate to the reaction at different concentrations (10 ng, 25 ng, 40 ng, and 80 ng). Additionally, we performed the HRM-PCR assay for CAT25 instability detection using DNA from 11 tumours with MSI-High on three Real-time PCR instruments with HRM ability: ECO, RG, and Z480.

For HRM-PCR validation and testing of the allelic repeat pattern of CAT25, a fluorescent PCR-based assay followed by capillary electrophoresis analysis (ABI3730XL instrument, Applied Biosystems, Foster City, USA) was performed. To amplify the CAT25 marker, SmallCAT25 primers with the labelled (FAM) forward primer and the same cycling cited for HRM-PCR were used. Capillary electrophoresis assay was performed by Macrogen (Seoul, Korea). We used Peak Scanner Software 2 (Thermo Fisher Scientific, Waltham MA, USA) to estimate the amplicon size. Procedure time (approximate): 120 minutes. To amplify BAT25 and BAT26 markers, we used primers and PCR cycling as previously described [14]. Forward primers were labelled with FAM (BAT25) and HEX (BAT26) fluorophores. Capillary electrophoresis analysis was performed following the same procedure as for CAT25.

Immunohistochemistry was performed on 9 biopsy samples classified as MSI-High. Samples were fixed in 10% pH 7 buffered formaldehyde before being embedded in paraffin, and then 5 microns

sections were made from selected blocks. The primary antibodies used were: MSH2 (CELL MARQUE, clone 6219–1129); MSH6 (CELL MARQUE, clone 44); MLH1 (Ventana, clone M1) and PMS2 (Ventana, clone ERP3947). The technique was carried out on Benchmark GX – VENTANA platforms (Roche, Mannheim, Germany) and revealed with Optiview DAB Detection Kit (Roche, Mannheim, Germany) in all cases. For the markings of MLH1 and PMS2, Optiview Amplification Kit (Roche, Mannheim, Germany) was also used according to protocols suggested by the manufacturer. Subsequently, the slides with the samples were dehydrated and mounted for visualization and evaluation under an optical microscope. A sample was considered negative when no tumour cell nucleus with positive staining for the protein under study (MSH2, MSH6, MLH1 or PMS2) was observed. Positive staining from the proteins studied observed in the cell nuclei of infiltrating lymphocytes, stromal cells and normal mucosa was considered as positive internal controls. "CAT25, BAT25 or BAT26 instability" was considered when a different amplicon size for each marker by capillary electrophoresis analysis was found comparing tumour DNA *vs*. peripheral blood DNA of the same patient. In HRM-PCR analysis, CAT25, BAT25 and BAT26 instability was considered to be present when HRM curves (Normalized and Different plot) were discordant between the samples previously described for capillary electrophoresis analysis.

Tumour samples were considered to have MSI-High when at least two of three mononucleotide markers (BAT25, BAT26, CAT25) showed instability by capillary electrophoresis analysis [10,11,21]. Predictive values and Fisher's exact test were calculated using GraphPad Prism 5 for Windows (GraphPad Software, Inc, San Diego, CA).

## Results

The HRM-PCR for CAT25 MSI analysis generated only one specific product with an adequate cycle threshold (CT) for HRM analysis (CT<30). The test reached 100% sensitivity and specificity for CAT25 since there was full concordance between the capillary electrophoresis and HRM-PCR results obtained with the analysis of 110 tumours and the paired peripheral blood from patients with colorectal cancer.

Different amounts of DNA template from a MSI-High tumour/peripheral blood (PB) ranging from 10 to 80 ng/reaction were studied. The 10 to 40 ng/reaction range provided a better interpretation of HRM curves, so that 25 ng/reaction was chosen for the following runs to be performed (Figure 1(a,b)). We found the same results for the 11 tumours with CAT25 instability in the three thermocyclers used. The reaction was reproducible in the intra-run precision–repeatability assay since the replicates grouped and melting profiles were equal for each sample in all 10 replicates (Figure 1(c,d)). In addition, there was no misinterpretation of HRM curves when the same procedure was performed on different days (Inter-run precision–reproducibility assay).

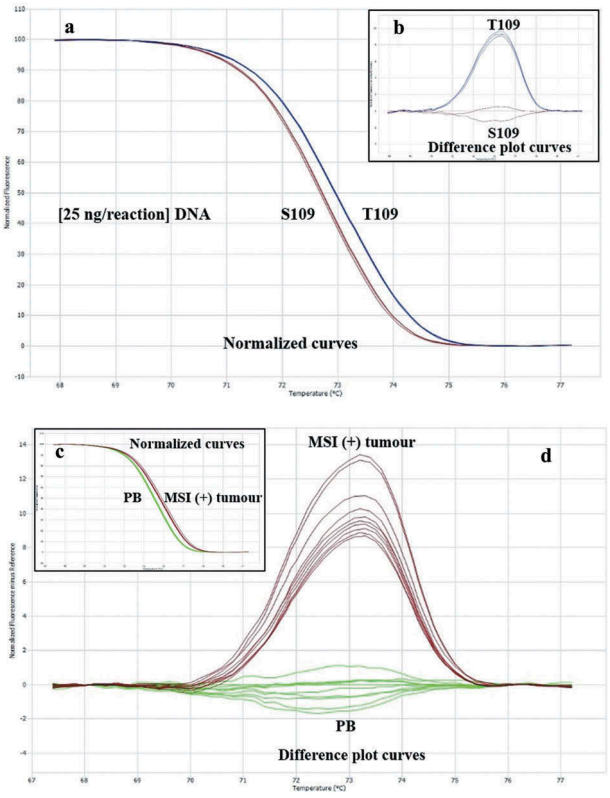


Figure 1. Variation of the amount of DNA template. From a range of 10 to 80 ng of DNA, 25 ng/reaction were chosen to conduct our study. (a) Normalized and (b) Difference plots HRM curves of CAT25 marker from DNA of a tumour with instability in CAT25 (T109) and peripheral blood (S109) from the same patient; both replicated three times. Intra-assay reproducibility test for CAT25 marker. (c) Normalized and (d) Difference plots HRM curves from DNA of MSI(+) tumour *vs*. DNA from peripheral blood (PB) from the same patient; both replicated ten times. MSI(+): tumour with instability in CAT25 marker proved by capillary electrophoresis. HRM: high resolution melting.

CAT25, BAT25 and BAT26 instability and predictive values data is as follows. The results obtained by Capillary electrophoresis analysis confirmed the HRM-PCR outcomes and made it possible to distinguish the differences in bp between the study samples (Figure 2). Taking into account the results of the three microsatellite markers studied, 11 of the 110 tumours analysed were classified as MSI-High. The instability of the microsatellites analysed by capillary electrophoresis, demonstrated the base pair shift, which in all cases was shown as shortening the length of the markers. The averages and standard deviations of these shifts in base pairs for each marker can be visualized in Table 1. The altered tumours displayed shifted HRM curves *vs*.

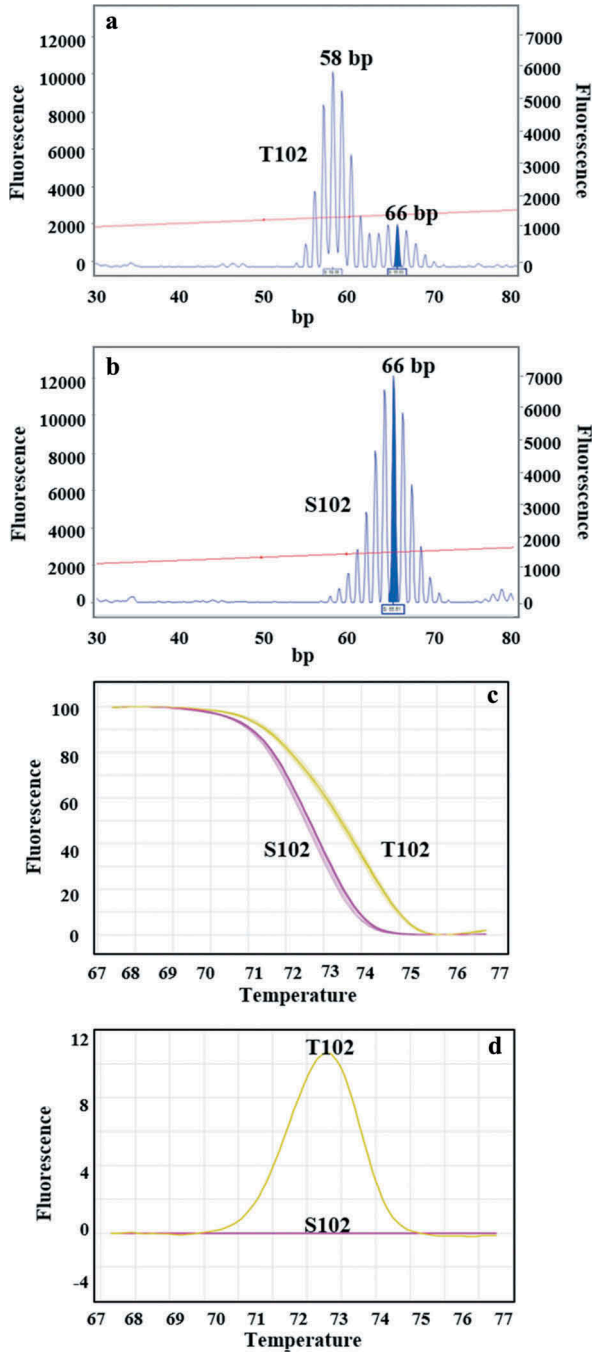


Figure 2. Comparative analysis between capillary electrophoresis and HRM techniques. Capillary electrophoresis shows a − 8 bp shift between DNA of tumour T102 (a) *vs.* DNA of peripheral blood (PB) S102 (b) from the same patient, denoting CAT25 instability. (c, d) Shifted allele in the cited samples reflected by normalized and difference plot HRM curves, respectively. HRM: high resolution melting; bp: base pairs.

those generated by PB from the same patient (Figure 2). Eleven samples from 110 colorectal tumour tissue presented allelic variation for CAT25, showing 100% positive predictive values (PPV) (95% CI, 71.5 to 100) and negative predictive values (NPV) (95% CI, 96.3 to 100) ($p < 0.001$) for MSI-High classification, using either HRM or capillary electrophoresis analysis.

Ten and 13 samples of the 110 cancers presented allelic variation for BAT25 and BAT26, respectively. One false positive for MSI-high classification was obtained for both BAT25 (tumour from patient 82) and BAT26 (tumour from patient 107) (Table 1). These data showed PPV for MSI testing of 92% for both. Two and no false negative were obtained for BAT25 (tumours from patients 35 and 64) and BAT26, respectively (Table 1). They also displayed NPV of 98% and 100%, respectively.

Allelic pattern analysis of CAT25. DNA samples from the healthy control group (n = 208) and MSI-High tumours (n = 11) were studied by capillary electrophoresis analysis using SmallCAT25 primers. This analysis showed a tight allelic range for CAT25: 64–66 bp from the healthy control group, with mean [SD] 64.9 [0.55] bp, standard. The DNA from colorectal tumours yielded a range de 57–64 bp, with mean [SD] 60.3 [2.4] bp ($p < 0.01$).

One of 11 tumours with CAT25 instability MSI exhibited a major peak of 64 bp in the electropherogram; this value is within the normal range, but the major peak in its corresponding peripheral blood was 66 bp, demonstrating allelic instability. The HRM analysis identified correctly this tumour sample as abnormal.

The immunohistochemistry (IHC) analysis was carried out in 9 of the 11 tumours with MSI-High (Table 1). The PMS2 enzyme was absent in all samples and no expression of MLH1 was observed in 8/9, whereas MSH2 and MSH6 were expressed in all the tumours (Figure 3). All the cases analysed by IHC correlated correctly with the results found in the microsatellite instability analysis (Table 1).

## Discussion

MSI analysis is widely used in Lynch Syndrome screening, this being the most common cause of inherited colorectal cancer, accounting for 2%–4% of newly diagnosed cases and it is transmitted through an autosomal dominant pattern caused by germline mutations in the mismatch repair genes [22].

The suggested replacement of the dinucleotide microsatellites from Bethesda panel for mononucleotides in the MSI analysis [7], promotes the study of new microsatellites associated with Lynch Syndrome. Our data suggest that CAT25 appears as a promising marker because it showed a limited allelic size variation and besides, CAT25 could potentially give the same information alone as when used together with two additional markers from the Bethesda panel (BAT25 and BAT26). Moreover, CAT25 analysis can be carried out using a fast and simple technique such as HRM-PCR with certain advantages over capillary electrophoresis

Table 1. Comparative results from Capillary electrophoresis, HRM curves and IHC analysis.

| | CE bp tumour (bp tumour – bp PB) | | | HRM curves (tumour *vs.* PB) | | | Immunohistochemistry | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | CAT25 | BAT25 | BAT26 | CAT25 | BAT25 | BAT26 | MSH2 | MSH6 | MLH1 | PMS2 | MSI |
| 35 | 62 (−4) | 120 (0) | 109 (−7) | instability | stability | instability | nd | nd | nd | nd | high |
| 48 | 58 (−8) | 115 (−5) | 105 (−9) | instability | instability | instability | nd | nd | nd | nd | high |
| 55 | 59 (−7) | 115 (−4) | 109 (−7) | instability | instability | instability | positive | positive | negative | negative | high |
| 64 | 61 (−5) | 120 (0) | 106 (−10) | instability | stability | instability | positive | positive | negative | negative | high |
| 79 | 63 (−3) | 116 (−3) | 106 (−8) | instability | instability | instability | positive | positive | negative | negative | high |
| 82 | 66 (0) | 107 (−13) | 116 (0) | stability | instability | stability | nd | nd | nd | nd | non-high |
| 92 | 64 (−2) | 114 (−5) | 109 (−6) | instability | instability | instability | positive | positive | negative | negative | high |
| 102 | 58 (−8) | 109 (−10) | 104 (−11) | instability | instability | instability | positive | positive | positive | negative | high |
| 107 | 66 (0) | 119 (0) | 104 (−9) | stability | stability | instability | nd | nd | nd | nd | non-high |
| 108 | 57 (−9) | 113 (−7) | 103 (−12) | instability | instability | instability | positive | positive | negative | negative | high |
| 109 | 65 (−1) | 113 (−6) | 106 (−9) | instability | instability | instability | positive | positive | negative | negative | high |
| 115 | 62 (−4) | 111 (−10) | 109 (−6) | instability | instability | instability | positive | positive | negative | negative | high |
| 116 | 58 (−7) | 112 (−7) | 107 (−8) | instability | instability | instability | positive | positive | negative | negative | high |
| Media bp (MSI high only) | 61 (−5) | 113 (−5) | 107 (−8) | | | | | | | | |
| Standard deviation | 2,8 [2,7] | 3,5 [3,4] | 2,2 [2,0] | | | | | | | | |

CE: capillary electrophoresis; HRM: high resolution melting; IHC: Immunohistochemistry; MSI: microsatellite instability; PB: peripheral blood; bp: base pairs; nd: not determined.

analysis, the most commonly used technique for MSI analysis.

Eleven of 110 tumour samples (10%) had MSI-High, in agreement with the range of 8 to 20% already reported in the literature [23]. Each of these MSI-High cases presented altered curves for CAT25 when analysed with our novel and validated CAT25 HRM-PCR which also matched the results obtained with capillary electrophoresis analysis for CAT25. These findings together with the results from BAT25 and BAT26 in MSI testing from the same population reinforce the view that reducing the number of markers to be used from the Bethesda panel in the screening of Lynch Syndrome as proposed [4,10,11]. Furthermore, fewer markers for MSI analysis provide the advantage of decreasing the costs and technical complexity while preserving the clinical applicability of the obtained results. In fact, CAT25 alone demonstrated the same clinical utility as using CAT25 together with BAT25 and BAT26 markers with optimal predictive values. Our findings are supported by previous literature using capillary electrophoresis analysis, where the sensitivity and specificity for microsatellite instability testing of CAT25 as a single marker were found to be the same as those detected when using five markers (BAT-25, BAT-26, MONO-27, NR-21, and NR-24) [25]. However, it is known that HRM-PCR is faster and simpler than capillary electrophoresis and does not need post-PCR handling steps, therefore, reducing the harmful chance of contamination that can distort the results.

The CAT25 HRM-PCR validation was performed in the ECO, but the other platforms could also be used to obtain excellent performance defining CAT25 instability in tumours, reinforcing the robustness of the technique. Taking into account that the technical characteristics vary, greater resolution capacity could be obtained when using the Z480. This platform allows obtaining 25 reading acquisitions per °C in the HRM step in contrast to the 10 reading acquisitions per °C offered by ECO and RG, in addition to a more complete software platform to interpret the HRM results. IHC analysis customary for Lynch Syndrome found that MLH1 and PMS2 enzymes were absent while MSH2 and MSH6 were present in the tumours. The incorrect expression of the enzymes in the tumour tissue supports the lack of correction in the DNA replication errors, demonstrated by the shortening in the length of the microsatellites in the MSI-High colorectal cancers.

Although CAT25 HRM-PCR possess all the mentioned advantages, we have found three limitations for this technique: 1- lack of multiplex format, if this was to be implemented, it could become a more simple and high-throughput assay; 2- The need of PB DNA that contains the normal allele required to contrast with the tumour DNA, for HRM analysis; and 3- the arguable limited application of our CAT25 HRM-PCR assay for paraffin-embedded tumour samples. The first two limitations are inherent to the HRM-PCR since it is an assay that is not characterized by easily adopting a multiplex format and usually a normal control is needed for the interpretation of its results, but they have the potential to be improved. On the third limitation, the validation of the assay for paraffin-embedded tissue will remain as a step to develop in the future. We believe all of these limitations can be solved through strategies such as those applied in analysers like IdyllaTM MSI Assay (RUO) (Biocartis) that has a multiplex HRM-PCR format (7 novel markers), it can perform the study directly from paraffined-preserved tissue and does not require the analysis of paired normal tissue samples [25]. However, the CAT25 analysis done by this new promising technology remains to be tested.

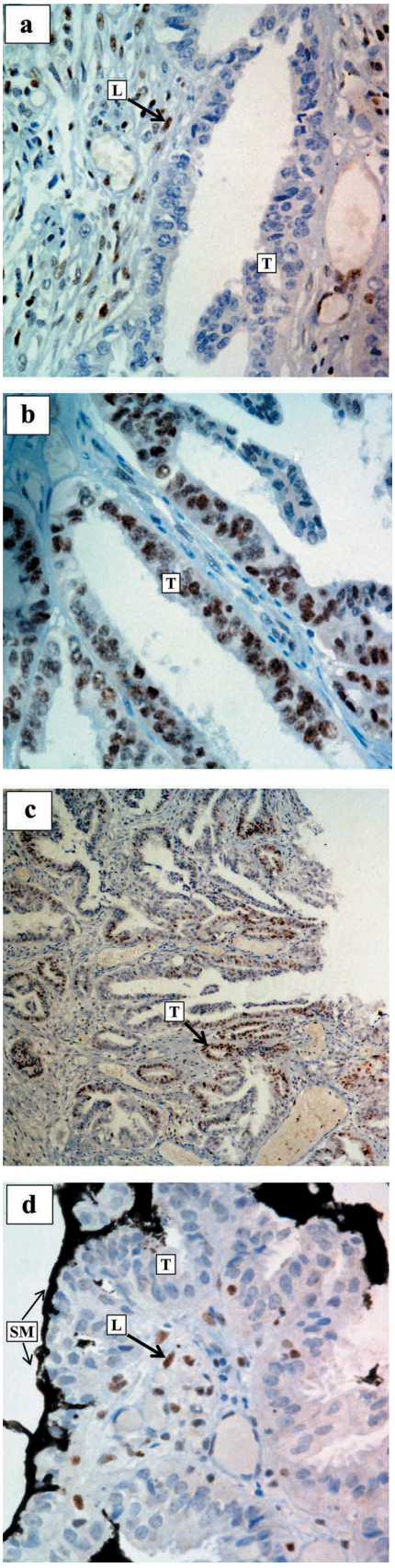


Figure 3. Immunohistochemical analysis. (a)- MLH1 40x, loss of nuclear MLH1 immunoreactivity in tumour tissue with immunoreactive lymphocyte infiltrate. (b)- MSH2 40x, positive nuclear immunoreactivity in tumour tissue. (c)- MSH6 10x, positive nuclear immunoreactivity in tumour tissue. (d)- PMS2 40x, Loss of nuclear PMS2 immunoreactivity in tumour tissue with immunoreactive lymphocyte infiltrate. T: tumour; L: lymphocyte; SM: surgical margin.

A clear caveat with our data is the small number of samples with MSI-High. Nevertheless, this work represents an advance in biomedical science because it provides pilot validation of an assay for CAT25 instability detection using PCR-HRM in colorectal cancer and supports the potential for CAT25 as a marker in MSI analysis.

## Summary table

*What is known about this subject*:
- MSI analysis is used in Lynch Syndrome screening.
- New mononucleotide microsatellites with limited size allelic variation are encouraged for the replacement of dinucleotides of the Bethesda panel.
- CAT25 is a clinically effective MSI analysis (by capillary electrophoresis) in different ethnicities.

*What this paper adds*:
- CAT25 instability can be detected by a quick and simple technique such as PCR-HRM with certain advantages over capillary electrophoresis.
- CAT25 has limited size allelic variation.
- CAT25 has the potential to be a good marker in the testing of MSI in colorectal cancer.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

## ORCID

AG Sánchez http://orcid.org/0000-0003-1927-3087
PA Romagnoli http://orcid.org/0000-0002-6328-9070

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