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ARTICLE

Stability of *BAT26* in tumours of hereditary nonpolyposis colorectal cancer patients with *MSH2* intragenic deletion

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Colon cancers arising in most patients with hereditary nonpolyposis colorectal cancer (HNPCC) show microsatellite instability (MSI). *BAT26*, a quasimonomorphic polyA stretch located just 3′ of *MSH2* exon 5, is considered the most sensitive and specific marker of MSI. A total of 10 HNPCC families with large intragenic *MSH2* deletions, encompassing exon 5 and intron 5, identified by multiplex ligation-dependent probe amplification (MLPA) were included in this study. The deletions under study were del1-16, del1-8, del1-7, del1-6, and del3-6, detected in 3, 1, 2, 3, and 1 families, respectively. Although all patients examined from these 10 families developed unstable tumours, 13/19 MSI-H tumours (68 %) surprisingly showed stability of *BAT26*. By MLPA and *MSH2* sequence analyses of the *BAT26*-stable tumours, we demonstrated that the wild-type *MSH2* allele was somatically inactivated by an identical large deletion, with complete loss of intron 5/*BAT26* sequences at the tumour DNA level. We could infer that the apparent stability of *BAT26* was due to the complete absence of target *BAT26* sequences in the tumour sample, which results in exclusive amplification of contaminant normal DNA, containing a single copy of a wild-type stable *BAT26* sequence. Identification of a subset of *MSH2*-related unstable tumours that are not recognized by analysis of *BAT26* instability indicates that this marker should never be used alone for rapid MSI screening of HNPCC tumours. Moreover, our findings indicate that *BAT26* stability in the context of MSI is strongly suggestive of the presence of a large intragenic *MSH2* deletion.
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Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC), the most common form of hereditary colon cancer, is an autosomal dominant syndrome characterized by a high incidence of early onset colorectal cancer and an excess of cancers of extracolonic sites, such as endometrium, small

intestine, urinary and hepatobiliary tracts, ovary, and stomach. [1,2]

HNPCC predisposition is caused by germline mutations in one of the known human mismatch repair (*MMR*) genes, namely *MLH1*, *MSH2*, *MSH6*, and *PMS2* (InSiGHT Mutation Database: http://www.insight-group.org/). Their inactivation may result from point mutations, identifiable in approximately 50–80% of the HNPCC families, or from genomic rearrangements, which can be detected in up to 20% of cases.[3–9] Large deletions are especially frequent in *MSH2*, and they account for up to one-third of *MSH2* mutations in some populations.[10]

Colon cancer arising in patients with a defective MMR system usually shows microsatellite instability (MSI), determined by the occurrence of frameshift mutations in simple sequence repeats. This molecular hallmark is useful for selecting patients and families eligible for *MMR* mutation analysis. To this aim, a standard panel of microsatellite markers for MSI testing of tumour tissues was proposed at the 1997 NCI international consensus meeting.[11] This panel is comprised of five markers, including two mono- (*BAT26* and *BAT25*) and three dinucleotide repeats (*D2S123, D5S346* and *D17S250*). Conventionally, all samples showing altered length in $\geqslant$2/5 markers are classified as MSI-H (high degree of instability), whereas samples with 1/5 (or less than 30%, if additional markers are tested) unstable markers and no altered markers are defined as MSI-L (low level of instability) and MSS (microsatellite stable), respectively.

More recently, an alternative panel of five mononucleotide repeats has been suggested.[12,13] This still includes *BAT26* and *BAT25*, the most widely used markers, which are deemed to be the most sensitive and specific markers for the detection of MSI-H tumours.[14–18]

BAT26 is a quasimonomorphic marker formed by a poly-A tract located immediately downstream of *MSH2* exon 5. For its high sensitivity, comprised between 93 and 100%, it has been proposed that it could be used as a single marker for MSI testing.[14,15,18–20] Here we show that HNPCC tumours from a subset of patients who are heterozygous for *MSH2* deletions are characterized by frequent stability of *BAT26*.

Materials and methods

Patients and tumours

A total of 10 HNPCC families were selected from a group of 16 families in which we previously identified large intragenic *MSH2* deletions by multiplex ligation-dependent probe amplification (MLPA) (Table 1). Overall, 18 deletions carriers from these 10 families, including the probands, were characterized by the inclusion of exon and intron 5 in the constitutionally deleted tracts. Lack of immunohistochemical MSH2 expression in tumours from nine out of nine tested families was consistent with the underlying *MSH2* genetic defect (data not shown). Some patients developed more than one tumour, and MSI status could be investigated on a total of 22 tumour DNA samples.

In the remaining six families with large *MSH2* deletions, exon 5/intron 5 were retained in the mutant allele. A total of nine tumours from six patients belonging to this subset were available for MSI analysis.

The research was approved by the local Ethical Committee and written consent was obtained from patients.

Table 1 *BAT26* status in MSI-H tumour samples from patients heterozygous for *MSH2* constitutional deletions

Family ID	*MSH2 genotype*[a]	*Sample ID*	*Tumour tissue*	*BAT26*[b]
A-AV62	del1-16[c]	CFS185	Colon–rectum	U
		CFS261	Stomach	S
			Kidney	U
		CFS262	Adenoma	U
		C459	Colon–rectum	U
A-AV90	del1-7[c]	CFS297	Colon–rectum	S
A-AV101	del1-7[c]	CFS337	Colon–rectum	S
A-AV111	del1-8[c]	CFS378	Adenoma	S
		CFS379	Colon–rectum	S
		CFS381	Colon–rectum	S
A-FI01	del1-16[c]	CFS406	Colon–rectum	S
A-FI02	del3-6[c]	CFS407	Colon–rectum	S
		CFS408	Colon–rectum	S
A-FI03	del1-16[c]	CFS424	Colon–rectum	S
A-VA25	del1-6[c]	CFS366	Colon–rectum	U
		CFS372	Colon–rectum	S
		CFS404	Colon–rectum	S
A-PD21	del1-6[c]	CFS356	Colon–rectum	U
A-PD29	del1-6[c]	CFS380	Colon–rectum	S
A-VA05	del8-10[d]	CFS236	Uterus	U
A-VA15	del7-8[d]	CFS303	Colon–rectum	U
			Adenoma	U
A-VA16	del8[d]	CFS316	Colon–rectum	U
A-FI04	del2-3[d]	CFS452	Colon–rectum	U
A-FI05	del7[d]	CFS454	Colon–rectum	U
A-FI06	del9-10[d]	CFS455	Colon–rectum	U

[a]Constitutional heterozygous *MSH2* deletion defined by MLPA.
[b]U, unstable; S, stable.
[c]Exon 5/intron 5 included in the deleted region.
[d]Exon 5/intron 5 not included in the deleted region.

MSI analysis

Genomic DNA was obtained from blood and from paraffin-embedded or frozen tissues. Tumour DNA was extracted by the DNAeasy tissue kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

Standard MSI analysis was performed on paired tumour–normal tissue DNA samples using the NCI panel of microsatellite markers (*BAT26, BAT25, D2S123*, *D5S346*, *D17S250*).[11] Where possible, additional markers were investigated (*NR21*, *NR22*, and *NR24*).[12] The fluorescently labelled PCR products were mixed with a size standard (GeneScan 400HD ROX, Applera, Foster City, CA, USA), run on an automatic ABI3100 DNA analyzer, and evaluated

with the GeneScan software (Applera). A variation in number and size of peaks of a marker in tumour DNA compared with normal DNA was interpreted as instability for that marker. Tumours were scored as MSI-H, MSI-L, or MSS, according to the international criteria.[11]

MLPA analysis

MLPA was performed with 200 ng of normal and tumour DNAs using the MRC-Holland (Amsterdam, Holland) HNPCC probe kit, according to the supplier's protocol. Then, 1 μl of the FAM-labelled PCR product was mixed with 1 μl of fluorescent GeneScan 500 LIZ size standard (Applera) in 15 μl of HiDi Formamide, run on an automatic ABI3100 DNA analyser, and evaluated with GeneScan software (Applera). The electropherograms showed specific peaks corresponding to each exon of *MSH2* and *MLH1*, as well as additional peaks corresponding to control sequences mapping on different chromosomes. Dosage analyses based on comparison between deleted and reference wild-type DNA samples were performed on an Excel file following the manufacturer's protocol (www.mrc-holland.com). Briefly, all *MSH2* peak areas were normalized by dividing each peak area by the combined area of all other peaks of the electropherogram. Then, these normalized peak areas were divided by the corresponding wild-type normalized peak area (average of three independent DNA samples) of that probe amplification product, in order to obtain a series of 16 a_{ex} values.

In constitutional and tumour DNA samples, a 40–55% decrease of the area of an *MSH2* exon peak compared to the wild-type control samples was considered as indicative of a heterozygous deletion of that exon. In tumor DNAs, a 60–90% decrease was considered as indicative of a homozygous deletion of that exon.

To quantitatively evaluate deletions in tumour DNAs, the following values were also calculated for each electropherogram of all tumour/normal DNA pairs: *Adel*, the mean value of normalized deleted *MSH2* peak areas; and *Aret*, the mean value of normalized retained *MSH2* peak areas, where present.

Polymorphism analysis

Four sequence polymorphisms of the *MSH2* locus were analysed: IVS1(+9)C/G, IVS9(−9)A/T, IVS10(+12)G/A, and IVS12(−6)A/G. Analysis was first performed by direct sequencing of PCR products on normal DNA. In heterozygous patients, the same polymorphism was evaluated on tumour DNA to check for the presence of loss of heterozygosity (LOH).

Results

MSI status was investigated on a total of 22 tumour DNA samples, including 15 colorectal carcinomas, two colorectal adenomas, two kidney tumours, and one tumour each of the stomach, bladder, and skin, from the 18 selected individuals with deleted exon 5/intron 5, and a total of nine tumours, including five colorectal carcinomas, two colorectal adenomas, and one tumour each of the uterus and skin, from the six patients with retained exon 5/intron 5. With the exception of the kidney tumour of patient CFS185 (family A-AV62), of the bladder and skin tumours of patient CFS366 (family A-VA25) and of the adenoma and skin tumour of patient CFS316 (family A-VA16), which were MSS (data not shown), all other tumours were clearly MSI-H. In particular, they all displayed instability at the *BAT25* marker. However, 13/19 tumours (68%) in the first group surprisingly showed stability of *BAT26*. This result was observed in tumours from nine different families, and was absent in four out of five tumours from family A-AV62 (del1-16), in one out of three tumours from family A-VA25 (del1-6), and in the only tumour of family A-PD21 (del1-6) (Table 1). *BAT26* was unstable in the seven MSI-H tumours from patients carrying *MSH2* deletions not including exon 5 (Table 1).

In order to gain some insight into the mechanisms underlying this unexpected phenomenon, we carried out additional molecular tests on tumour DNAs. As a first step, we used MLPA analysis to determine the copy number of each *MSH2* exon in tumour DNA samples. Owing to the poor yield and/or quality of some samples, only 13 tumour samples gave reliable results and could be used for tumour/constitutional comparisons. Careful visual inspection of the tumour electropherograms from the 10 *BAT26*-stable samples showed a further reduction in size of the same peaks deleted in matched normal DNA, without significant changes in the peaks that were not constitutionally deleted (Figure 1a). On the other hand, the electropherograms of the three *BAT26*-unstable tumours were undistinguishable from those of the corresponding constitutional DNAs (Figure 1b).

Precise qualitative and quantitative definition of these exonic deletions was carried out by dosage analysis of all single peaks. The exact extension of deletions in the different tumour/normal pairs was confirmed by calculating the a_{ex} values, which approximated $\sim$1 for the retained peaks, but were $\sim$0.5 or lower for the deleted peaks (Figure 2a and b). Moreover, to better define the borders of the somatically deleted regions, we verified that the flanking peak (ie the first retained peak immediately adjacent to the deleted region) had a normalized area of at least 85% of the expected value in each tumor DNA (data not shown).

Adel ranged from 0.45 ± 0.08 to 0.58 ± 0.09 in constitutional DNAs and from 0.18 ± 0.04 to 0.58 ± 0.13 in tumor DNAs (Figure 2c). However, in the *BAT26*-stable tumours, these values were in the range of 0.18 ± 0.04–0.37 ± 0.08 and, in each couple of tumor/normal DNA, the tumour *Adel* value was always significantly lower ($P < 0.001$) than the matched constitutional *Adel* value. On the contrary, *Adel* of the *BAT26*-unstable tumours had values between

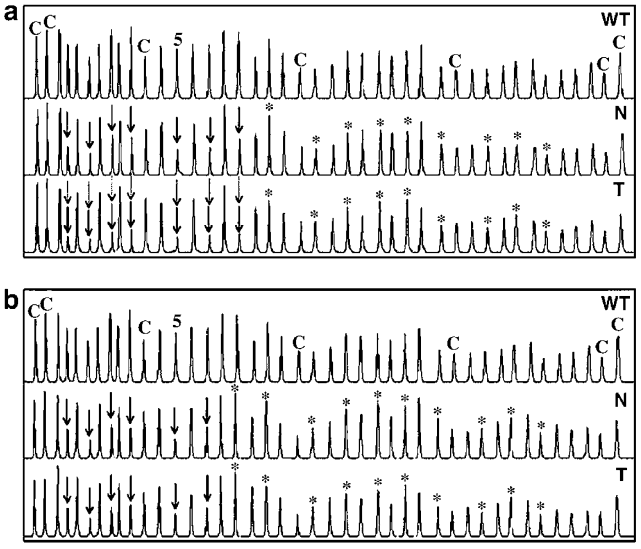

Figure 1 Large deletions of *MSH2* gene. Representative MLPA electropherograms of wild-type controls (wt) and constitutional (N) and tumour (T) DNAs from a *BAT26*-stable sample with del1-7 (**a**) and a *BAT26*-unstable sample with del1-6 (**b**). The MLPA control (C), *MSH2*-exon 5 (5), *MSH2*-deleted (↓), and *MSH2*-retained (*) peaks are indicated.

0.51 ± 0.05 and 0.58 ± 0.13, overlapping their corresponding constitutional *Adel* values (0.47 ± 0.08 and 0.56 ± 0.10).

Aret varied from 0.82 ± 0.12 to 1.08 ± 0.04 and from 0.80 ± 0.04 to 1.20 ± 0.04 in constitutional and tumour DNAs, respectively. The *Aret* values of the eight *BAT26*-stable tumours with retained exons were not dissimilar from the *Aret* value calculated for CFS356, the only *BAT26*-unstable sample retaining some *MSH2* exons (data not shown).

Overall, these data indicate that tumour somatic inactivation of the wild-type *MSH2* allele was determined by a second (intra)genic deletion, which was limited to the exons already deleted in constitutional DNA, in the *BAT26*-stable, but not in the *BAT26*-unstable tumour samples (Table 2).

Polymorphism analyses of some samples were not informative, due to constitutional homozygosity or to PCR difficulty in some paraffin-embedded samples. Only five tumours, four *BAT26*-stable and one unstable, could be evaluated for at least one of the four *MSH2* polymorphisms analysed. Heterozygosity was retained in the *BAT26*-unstable, but not in the four *BAT26*-stable tumour DNAs, which displayed total or partial LOH of *MSH2* sequence polymorphisms mapping outside the deleted region (Table 2).

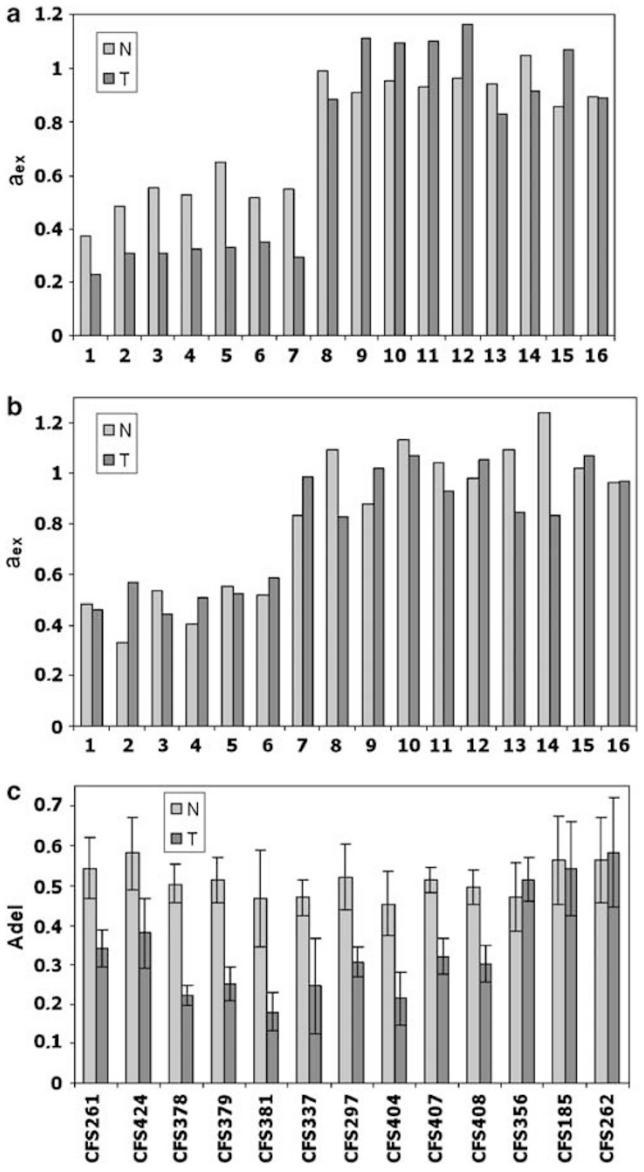

Figure 2 Dosage of deleted and retained exons. Representative histograms showing a_{ex} values of constitutional (N) and tumour (T) DNAs from a *BAT26*-stable sample with del1-7 (**a**) and a *BAT26*-unstable sample with del1-6 (**b**). Panel (**c**) shows *Adel* values in *BAT26* stable (from CFS261 to CFS408) and *BAT26* unstable (from CFS356 to CFS262) cases.

Discussion

This is the first study investigating the correlations between *BAT26* status and mutation type in *MSH2*. We observed an association between stability of the *BAT26* microsatellite marker and presence of large intragenic *MSH2* deletions encompassing exon and intron 5. Since *BAT26* stability was present in 13 out of 19 MSI-H tumours with such deletions, it appears to be a very frequent (68%) and specific phenomenon. In fact, *BAT26* was unstable in seven out of seven tumour samples from subjects who were heterozygous for *MSH2* deletions not encompassing exon and intron 5, and in our 10 year-laboratory experience,

Table 2 MLPA and LOH analyses of informative tumour DNAs

MSH2 del[a]	*Sample ID*	*Tumour tissue*	*BAT26*[b]	*MSH2 tumour MLPA*[c]	*MSH2 tumour polymorphisms*[d]
del1-16	CFS261	Stomach	S	del1-16/del1-16	n.i.
	CFS424	Colon–rectum	S	del1-16/del1-16	n.i.
del1-8	CFS378	Adenoma	S	del1-8/del1-8	L
	CFS379	Colon–rectum	S	del1-8/del1-8	L
	CFS381	Colon–rectum	S	del1-8/del1-8	NA
del1-7	CFS337	Colon–rectum	S	del1-7/del1-7	n.i.
	CFS297	Colon–rectum	S	del1-7/del1-7	n.i.
del1-6	CFS404	Colon–rectum	S	del1-6/del1-6	NA
del3-6	CFS407	Colon–rectum	S	del3-6/del3-6	L
	CFS408	Colon–rectum	S	del3-6/del3-6	L
del1-6	CFS356	Colon–rectum	U	del1-6/wt	H
del1-16	CFS185	Colon–rectum	U	del1-16/wt	n.i.
	CFS262	Adenoma	U	del1-16/wt	n.i.

[a]Heterozygous *MSH2* deletion defined on constitutional DNA by MLPA.
[b]S, stable; U, unstable.
[c]Heterozygous or homozygous *MSH2* deletion defined on tumour DNA by MLPA; wt, wild type.
[d]L, LOH; H, heterozygosity retained; n.i., not informative; NA, not available. Cumulative data derived from four intragenic polymorphisms are reported.

BAT26 stability was very rarely observed in tumour DNAs from patients with *MMR* gene point mutations and never observed in patients with large intragenic *MLH1* deletions. Only recently it has become evident that large rearrangements of *MMR* genes cause Lynch syndrome in about 20% of cases. MLPA, a new high-resolution, robust and quick assay for detecting copy number variations in genomic sequences, has provided an important contribution for the detection of several new *MSH2* and *MLH1* deletions in some cohorts of families.[4,5,8]

Owing to its high sensitivity and specificity, *BAT26* was initially proposed as an unambiguous marker of MSI,[14] and therefore it has been included in both microsatellite panels proposed at the first[11] and second[13] Bethesda consensus meetings. Recently, it has been reported that *BAT26*, together with *BAT25*, accurately detects MSI-H tumours and can also be used to predict tumour content (the percentage of tumour cells) in DNA samples.[18]

BAT26 is a quasimonomorphic poly-A stretch located just 3′ of *MSH2* exon 5. We hypothesized that *BAT26* stability could be due to somatic deletions, with complete loss of intron 5/*BAT26* sequences in tumour cells, and, consequently, amplification of a wild-type allele present in infiltrating leucocytes and/or stromal cells. By MLPA analysis, we demonstrated that the wild-type *MSH2* allele in *BAT26*-stable tumours, but not in the *BAT26*-unstable samples, is somatically inactivated by another large deletion encompassing intron 5/*BAT26* sequences, thus confirming our hypothesis. We found that MLPA is a very easy and efficient test to detect large *MSH2* rearrangements also in the majority of DNA samples extracted from paraffin-embedded tumours. Visual and quantitative evaluation of the MLPA peaks areas showed that normal and tumour DNAs had similar patterns in the *BAT26* unstable cases, with *Adel* values ≈ 0.5, as expected. On the other hand, the MLPA patterns of the *BAT26*-stable tumours were different from those of the corresponding normal tissues, showing on average $Adel < 0.5$ and $Aret \approx 1$. This suggests that somatic inactivation of the wild-type *MSH2* allele was determined by a second identical deletion, which leads to homozygosity of the constitutional mutation. However, only DNA sequencing of breakpoints on both alleles could definitively confirm this hypothesis. Hemizygosity, as alternative to homozygosity, was excluded because the height of retained *MSH2* peaks relative to the other internal peaks did not significantly vary between normal and tumour DNAs. However, reduction to homozygosity was never complete, showing smaller deleted peaks of variable height, likely due to the presence of contaminating normal DNA.

We then investigated the status of *MSH2* intragenic polymorphic markers in tumour/constitutional DNA pairs. Data from these polymorphisms were concordant with those obtained with MLPA analysis, showing the presence of two different alleles in the only informative *BAT26*-unstable tumour DNA sample (CFS356), and LOH in all four *BAT26*-stable samples, possibly due to the presence of two identical *MSH2* alleles. Two mechanisms can account for the reduction to homozygosity for *MSH2* deletions observed in the majority of the samples with a stable *BAT26* marker: loss of the wild-type chromosome with duplication of the whole chromosome 2 containing the mutant allele or, alternatively, a somatic recombination, possibly associated with DNA repair failure, with copy/duplication of a DNA tract restricted to a region of the short arm surrounding the *MSH2* locus. Further analyses will be necessary to discriminate between these two hypotheses.

Our findings indicate that *BAT26* stability in a context of MSI-H is strongly suggestive of the presence of a large

intragenic *MSH2* deletion, especially when supported by absence of MSH2 immunoistochemical expression and positive family history. In fact, 68% of MSI-H tumours developed by patients with different *MSH2* deletions encompassing exon/intron 5 were *BAT26* stable, because of a second hit leading the constitutional mutation to homozygosity. Only two deletions escaped this rule: del1-16 (*BAT26* instability in 4/7 MSI-H tumours) and del1-6 (two of the five samples examined were unstable). Although literature data on the types of somatic mutations in *MSH2*-related tumours are scanty, point mutations have also been reported in addition to allelic loss/LOH mutations.[21–24] Our data demonstrate that in a small subgroup of patients with specific *MSH2* constitutional mutations, LOH associated with homozygosity of the mutant allele is the preferred mechanism of somatic inactivation, and suggest that the first *MSH2* hit can drive the molecular events associated with the second hit.

Finally, our findings bear important consequences for the clinical setting. Assessment of MSI status is used for the diagnosis of HNPCC and may also be relevant for disease prognosis[25] and for predicting response to chemotherapy.[26] As a consequence of an increasing demand for this molecular test, it is very important to arrange rapid and cost-effective tests with panels of microsatellite markers able to satisfy both clinical requirements. Identification of a subset of *MSH2*-related MSI-H tumours that are not recognized by *BAT26* indicates that this marker should never be used alone for the evaluation of instability in a rapid MSI screening of HNPCC tumours.

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