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Evaluation of a new panel of six mononucleotide repeat markers for the detection of DNA mismatch repair-deficient tumours

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Background: Microsatellite instability (MSI) is a molecular phenotype due to defective DNA mismatch repair (MMR) system. It is used to predict outcome of colorectal tumours and to screen tumours for Lynch syndrome (LS). A pentaplex panel composed of five mononucleotide markers has been largely recommended for determination of the MSI status. However, its sensitivity may be taken in default in occasional situations. The aim of the study was to optimise this panel for the detection of MSI.

Methods: We developed an assay allowing co-amplification of six mononucleotide repeat markers (BAT25, BAT26, BAT40, NR21, NR22, NR27) and one polymorphic dinucleotide marker (D3S1260) in a single reaction. Performances of the new panel were evaluated on a cohort of patients suspected of LS.

Results: We demonstrate that our assay is technically as easy to use as the pentaplex assay. The hexaplex panel shows similar performances for the identification of colorectal and non-MSH6-deficient tumours. On the other hand, the hexaplex panel has higher sensitivity for the identification of MSH6-deficient tumours (94.7% *vs* 84.2%) and MMR-deficient tumours other than colorectal cancer (92.9% *vs* 85.7%).

Conclusion: The hexaplex panel could thus be an attractive alternative to the pentaplex panel for the identification of patients with LS.

Instability of short tandem repeats or microsatellite instability (MSI) is a molecular phenotype due to defective DNA mismatch repair (MMR) system. About 15% of sporadic colorectal cancers, about 20% of sporadic endometrial cancers, as well as a non-negligible proportion of cancers originating from other sites exhibit MSI (Ionov *et al*, 1993; Thibodeau *et al*, 1993; Salovaara *et al*, 2000). A MSI phenotype is also observed in most tumours from patients with Lynch syndrome (LS; Aaltonen *et al*, 1993; Salovaara *et al*, 2000; Lynch *et al*, 2009). At the clinical level, patients with MSI colorectal tumours are known to have better stage-adjusted prognosis and may respond differently to adjuvant chemotherapy (Gryfe *et al*, 2000; Ribic *et al*, 2003; Sargent *et al*, 2010).

LS (MIM# 120435, 609310), also known as hereditary non-polyposis colorectal cancer (CRC), is the most common cause of

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inherited CRC. It is caused by germline mutations in MMR genes, not only affecting mainly *MLH1* and *MSH2* (∼40% each) but also *MSH6* and *PMS2* (∼10% and 5%, respectively). Patients with LS are at high risk of developing early-onset CRC. Patients with LS are also at risk of developing other primary cancers involving endometrium, ovary, urinary tract, stomach, small intestine, hepatobiliary tract, skin, and brain (Vasen *et al*, 2007; Lynch *et al*, 2009; Bonadona *et al*, 2011). The young age of disease onset (average before 45 years) highlights the importance of identifying these patients. Tumour testing for the presence of MMR deficiency has been recognised to be a relevant screening method to identify patients with MMR germline mutations (Umar *et al*, 2004).

Various repeat markers have been proposed to determine MSI. In 1997, an international meeting at the National Cancer Institute recommended the use of a panel of five markers (three dinucleotide and two mononucleotide repeats), known as the Bethesda panel (Boland *et al*, 1998). However, some limitations appeared, primarily due to the use of dinucleotide markers that showed lower sensitivity and specificity compared with mononucleotide markers (Perucho, 1999; Suraweera *et al*, 2002). In 2002, Hamelin and collaborators proposed a new panel of five quasi-monomorphic mononucleotide markers, known as the pentaplex panel, which was shown to allow accurate identification of MSI tumours without the need of matched normal DNA (Suraweera *et al*, 2002). This panel showed increased sensitivity and specificity compared with the Bethesda panel and was thus recommended for MSI testing (Buhard *et al*, 2004; Umar *et al*, 2004; Wong *et al*, 2006; Xicola *et al*, 2007; Goel *et al*, 2010; You *et al*, 2010). Nevertheless, in our experience, the pentaplex panel still failed detecting some tumours from patients with LS, especially tumours from patients with a germline *MSH6* mutation and/or tumours other than CRC.

The aim of the present study was to optimise the panel of microsatellite markers for detection of MSI and to evaluate the performances of this new panel for the identification of MMR-deficient tumours.

MATERIALS AND METHODS

Tissue specimens. A total of 148 tumours from patients with suspicion of LS were included in the study. Tumours and matched normal tissues when available ($n=120$) were obtained from Pathology Centers from North of France. These included 71 CRC and 7 colorectal adenomas, 31 endometrial carcinomas, 12 upper urinary tract carcinomas, 12 ovarian carcinomas, 7 skin tumours (6 sebaceous carcinomas and one adenoma), 6 gastric carcinomas, and 2 cerebral tumours.

Among these tumours, 77 were with defective MMR (dMMR) and 71 were with proficient MMR (pMMR) on the basis of MMR protein expression ($n=138$) and/or the presence of somatic *MLH1* hypermethylation or germline MMR mutation ($n=61$). The MSI status of the tumours was established using the original pentaplex panel (Suraweera *et al*, 2002). Fifty-eight tumours were found MSI-high (MSI-H) with instability at ⩾3 of the five markers. Fifteen additional tumours were found MSI-low (MSI-L) with instability at only one ($n=7$) or two ($n=8$) markers after comparison to normal DNA. Methods used were as described previously (Suraweera *et al*, 2002; Aissi-Ben Moussa *et al*, 2009; Crepin *et al*, 2011). Only patients with unambiguous results were included in the study. Full informed consent for the study was obtained from all the patients.

MSI analysis with the new panel. DNA was extracted from archival formalin-fixed paraffin-embedded (FFPE) tissue samples using the EZ1 DNA Tissue tissue kit with the BioRobot EZ1 (Qiagen, Courtaboeuf, France).

DNA samples isolated from tumours and corresponding normal tissues were tested for MSI using a set of six mononucleotide repeat markers: BAT25, BAT26, NR21, NR22 selected from the original pentaplex panel (Suraweera *et al*, 2002), NR27 selected from the modified pentaplex panel (Buhard *et al*, 2006), and BAT40, a polyT located on chromosome 1, which was shown to be particularly sensitive in both CRC and extra-colonic tumours (Hartmann *et al*, 2002; Kuismanen *et al*, 2002; Hendriks *et al*, 2004). The NR24 marker was not kept in the hexaplex panel because of its lower sensitivity (Goel *et al* 2010, and personal data). One highly polymorphic dinucleotide repeat marker (D3S1260) was added to the hexaplex as an internal control to check the correspondence between tumour DNA and matched normal DNA.

Primers and fluorescent markers were designed to allow efficient amplification of DNA extracted from FFPE tumours and resolution of different amplified products. Primer sequences and fluorescent markers are given in Table 1.

The seven markers were co-amplified in one tube using the Multiplex Master Mix (Qiagen). PCR was carried out in a 25-μl final volume containing 1 × Multiplex Master Mix, 0.24 μM of each primer pair, except for NR27 (0.12 μM) and BAT40 (0.48 μM), and about 50 ng of DNA. The PCR conditions are described in details in Supplementary Methods. The amplified products were separated on an ABI Prism 3130XL analyser and analysed using GeneMapper analysis software (Applied Biosystems, Courtaboeuf, France). A microsatellite marker was considered unstable when its size differed by at least 2 bp compared with the germline DNA (matched normal DNA or quasi-monomorphic variation range obtained from normal tissues). All tumours with ⩾1 unstable markers were considered as having some degree of instability and designated as MSI. Tumours with instability at ⩾3 of the six mononucleotide markers compared with the germline DNA were defined as MSI-H. Tumours with instability at one or two markers compared with germline DNA were defined as MSI-L. The cutoff

Table 1. Primer sequences and allele sizes

Marker	Sense primer (5′→3′)	Antisense primer (5′→3′)	Label	Allele size[a] (bp)
BAT25	TCGCCTCCAAGAATGTAAGT	TCTGCATTTTAACTATGGCTC	HEX	122–124
BAT26	TGACTACTTTTGACTTCAGCC	AACCATTCAACATTTTTAACCC	6-FAM	116–117
BAT40	AGTCCATTTTATATCCTCAAGC	GTAGAGCAAGACCACCTTG	NED	142–143 and 145–146
NR21	TAAATGTATGTCTCCCCTGG	ATTCCTACTCCGCATTCACA	HEX	98–99
NR22	GAGGCTTGTCAAGGACATAA	AATTCGGATGCCATCCAGTT	6-FAM	138–140
NR27	AACCATGCTTGCAAACCACT	CGATAATACTAGCAATGACC	6-FAM	86–87
D3S1260	CTACCAGGGAAGCACTGTAG	CATGTACCTGAGCACCTACTG	6-FAM	171–189

[a]Most common alleles.

for classification was chosen on the basis of the threshold of about 30–40% that is commonly used to distinguish MSI-H and MSI-L samples (Tomlinson *et al*, 2002).

Statistical analyses. The performances of the different microsatellite markers for MSI detection was compared by evaluating the sensitivity, specificity, positive predictive (PPV), and negative predictive values (NPVs), which were calculated using standard definition. The statistical software GraphPad InStat, version 3.10 (La Jolla, CA, USA) was used to calculate the 95% confidence intervals.

Allele size variations were summarised using means and s.ds. and compared in groups by Mann–Whitney *U* test.

The cutoff distinguishing MSI tumours from MSS tumours was ⩾1 unstable marker except otherwise indicated.

A *P*-value <0.05 was considered as statistically significant. Statistical analyses were performed using the SPSS software version 15.0 (SPSS, Chicago, IL, USA).

RESULTS

Fluorescent heptaplex PCR. The panel consisted of six mononucleotide repeat markers: BAT25, BAT26, BAT40, NR21, NR22, and NR27, and a highly polymorphic dinucleotide repeat marker: D3S1260. Primers were designed to be less sensitive to DNA quality, and PCR conditions were defined to allow co-amplification of the seven microsatellite markers in a single heptaplex PCR.

Most common sizes for BAT25, BAT26, BAT40, NR21, NR22, and NR27, as observed with normal DNA samples, were 122–124 bp, 116–117 bp, 145–146 bp, 98–99 bp, 138–139 bp, and 86–87 bp, respectively. These sizes were particularly short, allowing efficient amplification of the six mononucleotide markers of interest from all the samples. Examples of profiles are given in Figure 1.

Size variations, as observed with normal DNA, were very infrequent for BAT25 (1.30%), BAT26 (0.85%), NR21 (1.28%), NR22 (0.42%), and NR27 (0.42%), indicating that these markers are highly monomorphic in our population. By contrast, BAT40 was more polymorphic. Alleles ranged from 122 bp to 150 bp, 142–143 pb and 145–146 pb being the most common alleles (28.7% and 59.1% of the observed alleles, respectively).

Out of the 148 tumours included in the study, 76 (51.4%) showed instability at ⩾1 markers. Sixty-six tumours (44.6%) were MSI-H with instability at ⩾3 markers and 10 (6.8%) were MSI-L with instability at 1 ($n=4$) or 2 ($n=6$) markers. Matched normal DNA was available for 120 tumours (81.1%). Of the 28 tumours without matched normal DNA, six showed uninterpretable markers due to the presence of uncommon alleles: one for BAT26, one for NR21, one for NR27, and three for BAT40. None of the 28 tumours displayed >1 uninterpretable marker. Notably, BAT40 profile was profoundly modified in most unstable tumours (Figures 1 and 2). Blinded analysis of BAT40 without reference to

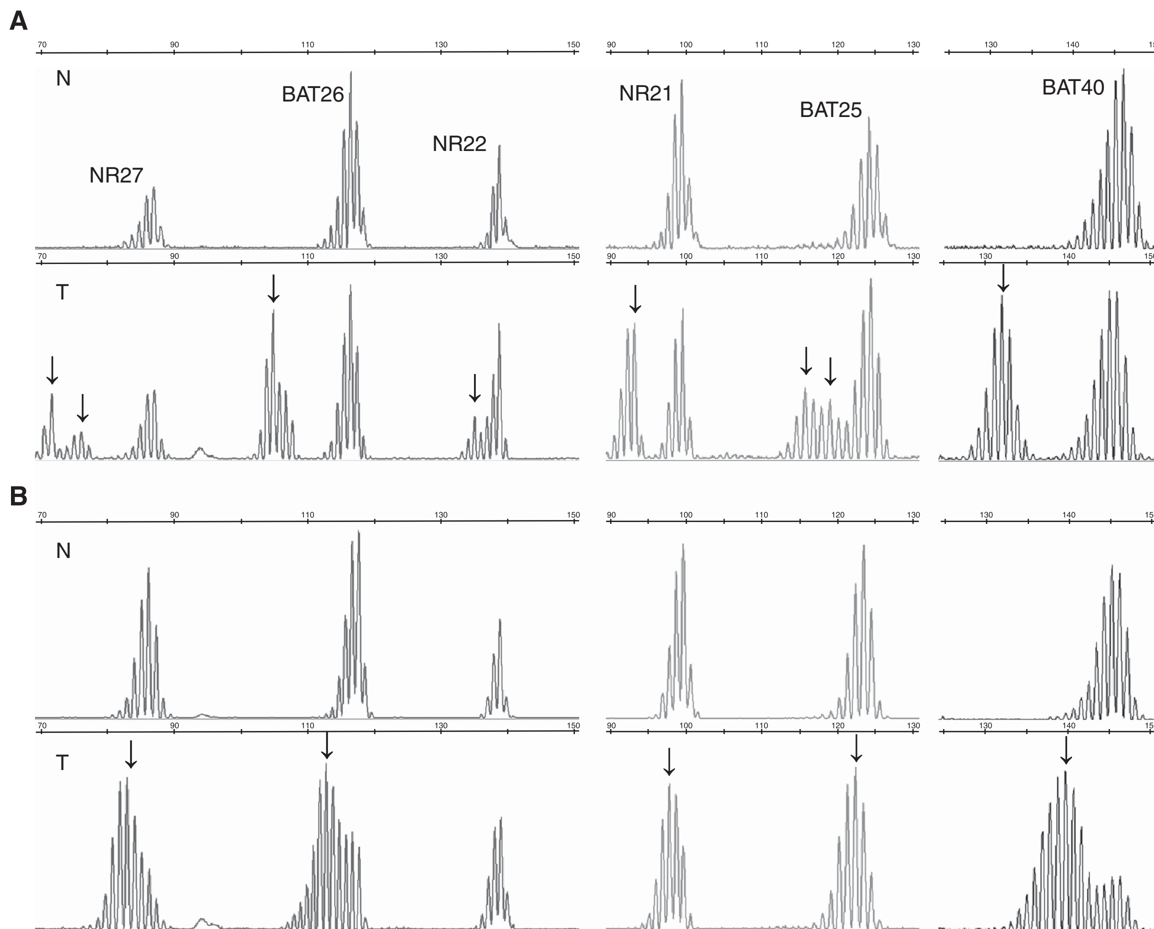

Figure 1. Representative examples of MSI profiles obtained with the hexaplex panel. MSI profiles in (**A**) colorectal and (**B**) endometrial dMMR tumours and matched normal tissues. (**A**) CRC from a patient with a *MLH1* germline mutation and loss of MLH1 and PMS2 expression, showing instability for the six microsatellite markers. (**B**) Endometrial tumour from a patient with a *MLH1* germline mutation and loss of MLH1 and PMS2 expression, showing instability for NR27, BAT26, NR21, BAT25, and BAT40. Additional alleles are indicated (arrows). Note the shorter allelic shifts in the endometrial tumour compared with the CRC. Abbreviations: N = normal tissue; T = tumour tissue.

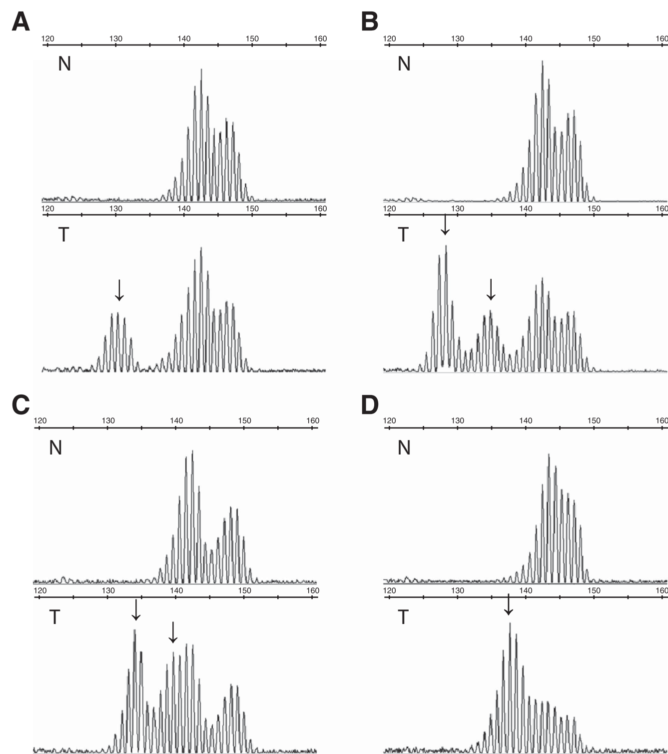

Figure 2. Representative examples of MSI profiles obtained with BAT40. MSI profiles in four dMMR tumours and matched normal tissues. Patients are heterozygous for BAT40. Profiles are profoundly modified in tumours compared with the germline DNA with one or multiple additional alleles (arrows). Abbreviations: N = normal tissue; T = tumour tissue.

Table 2. Characteristics of tumours ($n = 148$)

Tumours	dMLH1 ($n = 24$)	dMSH2 ($n = 30$)	dMSH6 ($n = 19$)	dPMS2 ($n = 4$)	dMMR ($n = 77$)	pMMR ($n = 71$)
CRC ($n = 71$)	18	8	7	2	**35**	**36**
Endometrial cancer ($n = 31$)	4	10	5	2	**21**	**10**
Urothelial cancer ($n = 12$)	1	4	1	0	**6**	**6**
Ovary cancer ($n = 12$)	0	2	3	0	**5**	**7**
Skin cancer ($n = 7$)	0	3	2	0	**5**	**2**
Gastric cancer ($n = 6$)	1	0	0	0	**1**	**5**
Colorectal adenoma ($n = 7$)	0	2	1	0	**3**	**4**
Brain cancer ($n = 2$)	0	1	0	0	**1**	**1**

Abbreviations: CRC = colorectal cancer; dMMR = defective DNA mismatch repair; pMMR = proficient DNA mismatch repair.
Bold faced numbers correspond to global effectives.
dMMR corresponds to the sum of dMLH1, dMSH2, dMSH6 and dPMS2.

matched normal DNA revealed that all tumours but one (147/148, 99.3%) would have been correctly classified in the absence of normal tissue due to the presence of >2 alleles (Figure 2A–C) or to an abnormal pattern of slippage that is not observed with germline DNA (Figure 2 D).

Screening performance of the hexaplex panel. To determine the performance of our assay, we investigated a series of 148 tumours from patients suspected of LS, which consisted of 77 dMMR tumours and 71 pMMR tumours based on the results of immunohistochemistry for MMR protein expression ($n = 138$), *MLH1* methylation analysis, and/or genetic testing ($n = 61$). Among dMMR tumours, 24 were MLH1-deficient (i.e., with combined loss of MLH1 and PMS2, somatic hypermethylation of the *MLH1* promoter, and/or germline mutation in *MLH1*), 30 were MSH2-deficient (with combined loss of MSH2 and MSH6 and/or germline mutation in *MSH2*), 19 were MSH6-deficient (with selective loss of MSH6 and/or germline mutation in *MSH6*), and 4 were PMS2-deficient (with selective loss of PMS2 and germline mutation in *PMS2*). Characteristics of the tumours are summarised in Table 2.

We evaluated the performance characteristics of the six mononucleotide repeat markers for identification of dMMR tumours. The panel displayed 85.7% sensitivity, 100% PPV, 100% specificity, and 86.6% NPV when instability at ⩾3 markers defined MSI. Corresponding values were 96.1% sensitivity and 97.4% PPV with 97.2% specificity and 95.8% NPV for a cutoff at one or more unstable markers (Table 3). Of the 77 dMMR tumours, 66 (85.7%) showed instability at ⩾3 markers and 8 (10.4%) showed instability at 1 ($n = 4$) or 2 ($n = 4$) markers (Figure 2). Tumours with low instability included one CRC, two endometrial tumours, three urothelial tumours, one ovary tumour, and one rectal adenoma. Characteristics of the tumours are summarised in Table 4 and Figure 3. Three tumours did not show instability at any of the six markers. These included two endometrial cancers, one of which was shown to contain <10% of viable tumour cells and one rectal adenoma with low-grade dysplasia. Notably, two out of the three dMMR adenomas tested showed some instability (with 4 out of 6 unstable markers in one case and 1 out of 6 in the other case). Of the 71 pMMR-classified tumours, 2 showed some instability using the hexaplex panel: 1 rectal adenoma and 1 sebaceous adenoma, both with instability at 2 markers (Table 4).

We next examined the performance characteristics of individual markers, with special focus on BAT40. The sensitivity and NPV were high for all markers, ranging from 64.1% and 71.4%, respectively, for NR22, to 92.2% and 91.7%, respectively, for BAT40 (Table 5). Six dMMR tumours showed stable BAT40, three of which were stable for all markers (Figure 3). Notably, one tumour with stable BAT40 showed instability at the five other markers. This tumour from a patient of African origin was homozygous for BAT40 with two unusual short 122 bp alleles.

The performance characteristics of the hexaplex panel were better for CRC than for non-CRC tumours regardless of the cutoff used for the MSI definition, with 100% sensitivity for CRC *vs* 92.9% for non-CRC tumours when ⩾1 unstable markers defined MSI (Table 6 and Supplementary Figure 1). As expected, the mean number of unstable markers per dMMR tumour was significantly reduced in non-CRC tumours (4.3 *vs* 5.4, $P = 0.001$) with a lower allelic shift when compared with matched normal DNA (mean total variation, 22.5 *vs* 39.5 bp, $P < 0.0001$) (Figure 3 and Supplementary Table 1). These differences were bigger for BAT40, NR27, and BAT26, contributing to the higher sensitivity of these markers in identifying non-CRC dMMR tumours.

The performance characteristics of the panel were very similar for MLH1-, MSH2-, or PMS2-deficient tumours and MSH6-deficient tumours, with 96.6% and 94.7% sensitivity, respectively, when ⩾1 unstable markers defined MSI (Table 6 and

Table 3. Performance characteristics of the hexaplex and pentaplex panels for the identification of dMMR tumours[a] ($n = 148$)

Panel	No. of unstable markers	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Hexaplex	≥3	85.7 (75.9–92.6)	100.0 (94.9–100.0)	100.0 (94.6–100.0)	86.6 (77.3–93.1)
	≥2	90.9 (81.9–96.2)	97.2 (90.2–99.7)	97.2 (90.2–99.7)	90.7 (81.0–95.6)
	≥1	96.1 (89.0–99.2)	97.2 (90.2–99.7)	97.4 (90.8–99.7)	95.8 (88.3–99.1)
Pentaplex	≥3	75.3 (64.1–84.4)	100.0 (94.9–100.0)	100.0 (93.8–100.0)	78.9 (69.0–86.8)
	≥2	85.7 (75.9–92.6)	100.0 (94.9–100.0)	100.0 (94.6–100.0)	86.6 (77.3–93.1)
	≥1	92.2 (83.8–97.1)	97.2 (90.2–99.7)	97.3 (90.5–99.7)	92.0 (83.4–97.0)

Abbreviations: CI = confidence interval; dMMR = defective DNA mismatch repair; NPV = negative predictive value; PPV = positive predictive value.
[a]Results are expressed as percentages, with 95% CIs in brackets.

Table 4. Clinical characteristics of dMMR tumours with low or absence of microsatellite instability and pMMR tumours with instability

Group	Site	Histology	Age (years)[a]	Criteria[b]	No. of unstable markers (type)	MMR protein expression	MMR germline/ somatic mutation	Remarks
dMMR	Colon	ADC	39	Beth	2/6 (BAT26, BAT40)	MSH6	*MSH6* c.2150_2153del (p.Val717Alafs)	+ Synchronous colon cancer
	Endometrium	ADC	49	Ams-II	1/6 (BAT26)	MLH1 PMS2	*MLH1* somatic hypermethylation	
	Endometrium	ADC	46	EC	1/6 (BAT40)	MSH6	*MSH6* c.3268_3272del (p.Glu1090Lysfs)	
	Endometrium	ADC	58	Ams-II	0/6	MSH6	*MSH6* c.3261delC p.Phe1088Serfs	
	Endometrium	ADC	57	Ams-I	0/6	N	*MLH1* c.1165C>A (p.Arg389*)	
	Ovary	ADC	49	Ams-II	2/6 (BAT40, NR22)	MSH6	*MSH6* c.2277_2281dup (p.Arg761Lysfs)	
	Urothelium	Carcinoma	48	Ams-I	2/6 (BAT26, NR27)	MLH1 PMS2	*MLH1* c.2117_2130del (p.Gly706Valfs)	
	Urothelium	Carcinoma	53	EC	2/6 (BAT40, NR22)	N	*MSH6* c.3080dupT (p.Ser1028Ilefs)	+ Endometrial carcinoma at 53
	Urothelium	Carcinoma	56	Ams-I	1/6 (BAT40)	MSH2 MSH6	*MSH2* c.1022T>C (p.Leu341Pro)[c]	+ Colorectal adenoma at 56
	Rectum	Adenoma	51	—	1/6 (BAT40)	MSH2 MSH6	*MSH2* c.793-2A>C (p.Val265_Gln314del)	+ Multiple sebaceous carcinomas at 51
	Rectum	Adenoma	56	Ams-I	0/6	N	*MSH2* c.1022T>C (p.Leu341Pro)[c]	+ Urothelial carcinoma at 56
pMMR	Skin	Sebaceous adenoma	54	EC	2/6 (BAT26, BAT40)	N	ND	+ Multiple sebaceous adenomas at <54 + past history of ovary carcinoma at 44
	Rectum	Adenoma	48	—	2/6 (BAT25, BAT40)	N	ND	

Abbreviations: ADC = adenocarcinoma; dMMR = defective DNA mismatch repair; EC = extended criteria; N = normal expression of the four DNA mismatch repair (MMR) proteins; ND = not determined; pMMR = proficient MMR.
[a]Age at diagnosis.
[b]Ams, Amsterdam criteria: Ams-I (Vasen *et al.*, 1991), Ams-II (Vasen *et al.*, 1999); Beth, revised Bethesda criteria (Umar *et al.*, 2004); EC Lynch Syndrome-related cancer <60 years (Olschwang *et al.*, 2004; French National Cancer Institute guidelines).
[c]Mutation c.1022T>C is classified as pathogenic (UMD MSH2 mutations database: http://www.umd.be/MSH2/).

Supplementary Figure 1). However, the mean number of unstable markers per dMMR tumour was significantly smaller in MSH6-deficient tumours compared with other MMR-deficient tumours (3.9 *vs* 5.3, $P = 0.001$) (Figure 3). Accordingly, the sensitivity of the panel was lesser for MSH6-deficient tumours compared with non-MSH6-deficient tumours (73.7% *vs* 88.1%) when the cutoff for MSI

dMMR tumours

ID n°	Site	Group	Protein
7279	Colon	CRC	MLH1
13164	Colon	CRC	MLH1
13188	Colon	CRC	MLH1
13996	Colon	CRC	MLH1
13827	Colon	CRC	MLH1
13990	Colon	CRC	MLH1
9889	Colon	CRC	MLH1
14229	Colon	CRC	MLH1
12970	Colon	CRC	MLH1
3942	Colon	CRC	MLH1
8432	Colon	CRC	MLH1
14092	Colon	CRC	MLH1
13089	Colon	CRC	MLH1
2270	Colon	CRC	MLH1
10290	Colon	CRC	MLH1
14261	Colon	CRC	MLH1
12385	Colon	CRC	MLH1
13910	Rectum	CRC	MLH1
3484	Endometrium	Non-CRC	MLH1
10269	Endometrium	Non-CRC	MLH1
7960	Endometrium	Non-CRC	MLH1
10663	Endometrium	Non-CRC	MLH1
1082	Stomach	Non-CRC	MLH1
7239	Urothelium	Non-CRC	MLH1
12968	Colon	CRC	MSH2
12738	Colon	CRC	MSH2
6673	Colon	CRC	MSH2
10344	Colon	CRC	MSH2
9892	Colon	CRC	MSH2
13047	Colon	CRC	MSH2
11968	Colon	CRC	MSH2
13037	Colon	CRC	MSH2
10942	Endometrium	Non-CRC	MSH2
6829	Endometrium	Non-CRC	MSH2
3079	Endometrium	Non-CRC	MSH2
6254	Endometrium	Non-CRC	MSH2
10204	Endometrium	Non-CRC	MSH2
3199	Endometrium	Non-CRC	MSH2
7356	Endometrium	Non-CRC	MSH2
10942	Endometrium	Non-CRC	MSH2
6617	Endometrium	Non-CRC	MSH2
7732	Endometrium	Non-CRC	MSH2
11086	Ovary	Non-CRC	MSH2
10942	Ovary	Non-CRC	MSH2
6099	Skin	Non-CRC	MSH2
1774	Skin	Non-CRC	MSH2
13463	Skin	Non-CRC	MSH2
1774	Brain	Non-CRC	MSH2
13216	Urothelium	Non-CRC	MSH2
13224	Urothelium	Non-CRC	MSH2
11688	Urothelium	Non-CRC	MSH2
9843	Urothelium	Non-CRC	MSH2
1774	Colon (adenoma)	Non-CRC	MSH2
9843	Rectum (adenoma)	Non-CRC	MSH2
13166	Colon	CRC	MSH6
13824	Colon	CRC	MSH6
14042	Colon	CRC	MSH6
11304	Colon	CRC	MSH6
14365	Colon	CRC	MSH6
1959	Colon	CRC	MSH6
8874	Colon	CRC	MSH6
4768	Endometrium	Non-CRC	MSH6
11757	Endometrium	Non-CRC	MSH6
13709	Endometrium	Non-CRC	MSH6
6112	Endometrium	Non-CRC	MSH6
14234	Endometrium	Non-CRC	MSH6
7297	Ovary	Non-CRC	MSH6
10804	Ovary	Non-CRC	MSH6
13942	Ovary	Non-CRC	MSH6
9405	Skin	Non-CRC	MSH6
7706	Skin	Non-CRC	MSH6
4706	Urothelium	Non-CRC	MSH6
11757	Rectum (adenoma)	Non-CRC	MSH6
11463	Colon	CRC	PMS2
11832	Rectum	CRC	PMS2
13617	Endometrium	Non-CRC	PMS2
12238	Endometrium	Non-CRC	PMS2

Markers: BAT40, BAT25, BAT26, NR21, NR22, NR27

pMMR tumours

ID n°	Site	Group
9232	Rectum (adenoma)	Non-CRC
7073	Skin (adenoma)	Non-CRC

Markers: BAT40, BAT25, BAT26, NR21, NR22, NR27

Figure 3. **Performance characteristics of hexaplex markers in dMMR tumours and MSI-positive pMMR tumours.** Black scares indicate unstable markers; white scares indicate stable markers.

definition was increased to three unstable markers (Supplementary Figure 1). Notably, allele size variations were bigger for BAT40, BAT26, BAT25, and NR27, suggesting higher sensitivity of these markers in identifying MSH6-deficient tumours (Supplementary Table 1).

Comparison of screening performance between the hexaplex and pentaplex panels. We compared the data obtained with our panel and with the original pentaplex panel composed of BAT25, BAT26, NR21, NR22, and NR24 markers on the same tumours ($n = 148$). As shown in Table 6 and Supplementary Figure 1, performances of the two panels were comparable for CRC-dMMR and non-MSH6-deficient tumours. But interestingly, the sensitivity of the hexaplex was higher for non-CRC-dMMR tumours and MSH6-deficient tumours, irrespective of the cutoff used for MSI definition (92.9% *vs* 85.7% and 94.7% *vs* 84.2%, respectively, when $\geqslant 1$ unstable markers defined MSI).

DISCUSSION

Determination of the MSI status is routinely performed as a screening test for LS. A pentaplex panel has been recognised as

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Table 5. Performance characteristics of individual markers for the identification of dMMR tumours[a] ($n=148$)

Marker	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
BAT25	74.4 (63.6–82.8)	98.6 (91.6–100.0)	98.3 (90.2–100.0)	77.5 (67.8–85.0)
BAT26	84.6 (74.9–91.1)	98.6 (91.5–100.0)	98.5 (91.3–100.0)	85.0 (75.4–91.4)
BAT40	92.2 (83.7–96.7)	97.1 (89.3–99.8)	97.3 (90.0–99.8)	91.7 (82.7–96.4)
NR21	70.1 (59.1–79.2)	100.0 (93.7–100.0)	100.0 (92.1–100.0)	75.0 (65.2–82.8)
NR22	64.1 (53.0–73.9)	100.0 (93.8–100.0)	100.0 (91.5–100.0)	71.4 (61.8–79.5)
NR27	82.1 (72.0–89.1)	100.0 (93.7–100.0)	100.0 (93.2–100.0)	83.1 (73.5–89.8)

Abbreviations: CI = confidence interval; dMMR = defective DNA mismatch repair; NPV = negative predictive value; PPV = positive predictive value.
[a]Results are expressed as percentages, with 95% CIs in brackets.

Table 6. Performance characteristics of the hexaplex and pentaplex panels for the identification of non-CRC-dMMR and MSH6-deficient tumours[a,b] ($n=148$)

	Hexaplex		Pentaplex	
Tumour	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
CRC	100.0 (90.0–100.0)	100.0 (90.3–100.0)	100.0 (90.0–100.0)	100.0 (90.3–100.0)
Non-CRC	92.9 (80.5–98.5)	94.3 (80.9–99.3)	85.7 (71.5–94.6)	94.3 (80.9–99.3)
Non-MSH6	96.6 (88.1–99.6)	—	94.8 (85.6–98.9)	—
MSH6	94.7 (74.0–99.9)	—	84.2 (60.4–96.6)	—
All	96.1 (89.0–99.2)	97.2 (90.2–99.7)	92.2 (83.8–97.1)	97.2 (90.2–99.7)

Abbreviations: CI = confidence interval; CRC = colorectal cancer; dMMR = defective DNA mismatch repair.
[a]Results are expressed as percentages, with 95% CIs in brackets
[b]Tumours showing instability for ⩾1 markers were considered as MSI.

very sensitive and specific for determination of MSI status and also easy to use (Buhard *et al*, 2004; Wong *et al*, 2006; Xicola *et al*, 2007; Goel *et al*, 2010; You *et al*, 2010). This led to its widespread recommendation and adoption (Umar *et al*, 2004; Xicola *et al*, 2007; Goel *et al*, 2010). However, a lower sensitivity of MSI testing has been described for some extra-colonic tumours and some MSH6-deficient tumours (Wu *et al*, 1999; Hartmann *et al*, 2002; Kuismanen *et al*, 2002; Hendriks *et al*, 2004). This contributes to an under-diagnosis of LS. We further optimised this panel for detection of MMR deficiency by adding a sixth mononucleotide marker, BAT40, which appeared to be particularly sensitive in extra-colonic tumours (Hartmann *et al*, 2002; Kuismanen *et al*, 2002; Hendriks *et al*, 2004). One highly polymorphic dinucleotide repeat marker was also added to the panel as an internal control to check the correspondence between tumour DNA and matched normal DNA when the latter is available. Primers and PCR conditions were determined to allow amplification of all markers in a single multiplex PCR, rendering the panel as simple to use as the pentaplex panel. BAT25, BAT26, NR21, NR22, and NR27 were highly monomorphic in germline DNA, in accordance with previous data obtained from a wide spectrum of population worldwide (Buhard *et al*, 2006). BAT40 was more polymorphic, with two common alleles (142–143 bp and 145–146 bp) accounting for 87.8% of the observed alleles. This implies that matched normal DNA would be necessary in a non-negligible proportion of cases for its interpretation. However, even in cases of heterozygosity without matched normal DNA for comparison, its profile is *per se* a good indicator of the real presence/absence of instability. Thus, matched normal DNA would remain not mandatory in most cases.

We validated the utility of the hexaplex for MSI detection in a large series of MMR-deficient and -proficient tumours. Our series was voluntarily enriched with non-CRC tumours as well as MSH6-deficient tumours that have been reported to be associated with a lower proportion of unstable markers and a smaller size of allelic shifts resulting in some false-negative results (Wu *et al*, 1999; Hartmann *et al*, 2002). We also chose to include adenomas in our study, because benign tumours are known to present a lower frequency and degree of instability when compared with malignant tumours, which may therefore be challenging to detect (Ferreira *et al*, 2009).

Our assay demonstrated high sensitivity with instability in 74 out of 77 (96.1%) dMMR cases and high instability (⩾3 markers) in 66 (85.7%) cases. Of note, three tumours (one endometrium, one urothelium, and one colonic adenoma) would have been missed and five additional tumours with a MSI-low phenotype (two colon, one ovary, and two urothelium) would have been also considered as MSS if the BAT40 marker had not been tested. This demonstrates the interest of this marker to identify dMMR tumours. In addition, allele size variations were bigger for BAT40, and therefore easier to detect, which should facilitate the interpretation in cases with slight slippage.

At the same time, our assay demonstrated two apparently 'false-positive' cases. These included one rectal adenoma and one sebaceous adenoma with two out of six unstable markers and maintained expression of MLH1, MSH2, MSH6, and PMS2 proteins. Of note, these tumours were also positive (with 1 out of 5 unstable marker) using the pentaplex panel. Unfortunately, blood samples were not available for *MMR* gene analysis for these patients. Such analysis may have revealed a defect of IHC in detecting MMR deficiency, as observed for two other cases in our series. The first one was an urothelial cancer that showed low MSI and maintained expression of the four MMR proteins and was

subsequently found associated with a *MSH6* pathogenic germline mutation (c.3080dupT); the second one was an endometrial cancer that showed high MSI and maintained MMR protein expression but was associated to a *PMS2* germline mutation (c.1831dupA).

Cutoffs regarding the number of unstable markers for the classification of MSI have been suggested to minimise false-positive results due to non-specific slippage or heterozygosity when matched normal DNA is missing. LS has been excluded or considered very unlikely in MSI-L cases (Umar *et al*, 2004). In addition, only MSI-H tumours are believed to have distinctive clinical features, including better prognosis (Halford *et al*, 2002; Laiho *et al*, 2002). Nevertheless, MSI-L has been shown to occur as a real phenomenon in colorectal, endometrial, and ovarian cancers, which are all LS-related cancers (Halford *et al*, 2003). Moreover, it has been shown recently that defining tumours showing instability at $\geqslant 2$ markers as MSI (instead of $\geqslant 3$ out of 5) considerably enhanced the screening performance of the pentaplex panel for MSH6-deficient tumours (Goel *et al*, 2010). In our series, the statistical analysis of the hexaplex panel performances revealed even better sensitivity when $\geqslant 1$ unstable markers defined MSI, with no loss of specificity. Eight dMMR tumours showed instability at <3 markers, including four tumours with instability at only one marker. Seven out of them were from patients carrying a pathogenic germline MMR mutation (4 *MSH6*, 2 *MSH2*, and 1 *MLH1*), indicating that the detection of this class of tumours is useful for LS identification.

It is particularly true for MSH6-deficient tumours that are expected to display lower instability due to its biological function. Indeed, MSH6 is preferentially involved in repair of single base pair mismatches and small insertion/deletion loops. Moreover, its repair function can partially be compensated by MSH3 (Drummond *et al*, 1995; Palombo *et al*, 1995; Acharya *et al*, 1996). This phenomenon has also been well demonstrated in *MSH6*-mutant mice (Edelmann *et al*, 1997).

It is also particularly relevant for extra-colonic tumours such as endometrial tumours that have been described to follow a specific tumourigenic pathway, leading to a lower proportion of unstable markers with shorter allelic shifts, and thus to a frequent MSI-low or MSS phenotype (Wijnen *et al*, 1999; Wu *et al*, 1999; Kuismanen *et al*, 2002; Wong *et al*, 2006). Of note, endometrial cancer is the most common extra-colonic cancer in LS, with an estimated cumulative risk of 35–60% for female mutation carriers, that may reach up to 71% for *MSH6* mutation carriers at 70 years of age (Hendriks *et al*, 2004; Vasen *et al*, 2007; Bonadona *et al*, 2011). This should be taken in consideration in the context of LS screening. Similarly, other tumours from patients with LS, such as urothelial tumours, brain tumours, or colorectal adenomas have been described to be frequently associated with a MSI-low or MSS phenotype (Hartmann *et al*, 2002; Gylling *et al*, 2008; Ferreira *et al*, 2009; Giunti *et al*, 2009). Because of the smaller size of allelic shifts observed in these tumours, we recommend to maintain analysis of matched germline DNA for routine-practice MSI screening of these cancer types.

In conclusion, this study further confirms the utility of routine molecular screening for LS in every type of potentially LS-associated tumours. Moreover, given the comparable advantages of our hexaplex panel in terms of rapidity and simplicity to the pentaplex one and its superiority in identifying some non-CRC dMMR and some MSH6-deficient tumours, we think that it is a good alternative screening test to identify patients with LS.

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