Molecular pathology of mismatch repair deficient tumours with emphasis on immune escape mechanisms

Dierssen, J.W.F.

Prediction of a mismatch repair gene defect by microsatellite instability and immunohistochemical analysis in endometrial tumours from HNPCC patients

Wiljo J. F. de Leeuw, Jan Willem Dierssen, Hans F. A. Vasen, Juul Th. Wijnen, Gemma G. Kenter, Hanne Meijers-Heijboer, Annette Brocker-Vriends, Astrid Stormorken, Pal Moller, Fred Menko, Cees J. Cornelisse, Hans Morreau

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1. ABSTRACT

Instability of microsatellite repeat sequences has been observed in colorectal carcinomas and in extracolonic malignancies, predominantly endometrial tumours, occurring in the context of hereditary non-polyposis colorectal cancer (HNPCC). Microsatellite instability (MSI) as a feature of human DNA mismatch repair (MMR)-driven tumourigenesis of the uterine mucosa has been studied primarily in sporadic tumours showing predominantly somatic hypermethylation of *MLH1*. The present study shows that all endometrial carcinomas (n=12) from carriers of *MLH1* and *MSH2* germline mutations demonstrate an MSI-high phenotype involving all types of repeat markers, while in endometrial carcinomas from MSH6 mutation carriers, only 36% (4 out of 11) demonstrate an MSI-high phenotype. Interestingly, an MSI-high phenotype was found in endometrial hyperplasias from *MSH2* mutation carriers, in contrast to hyperplasias from *MLH1* mutation carriers, which exhibited an MSI-stable phenotype. Instability of only mononucleotide repeat markers was found in both endometrial carcinomas and hyperplasias from *MSH6* mutation carriers. In 29 out of 31 (94%) endometrial tumour foci, combined MSI and immunohistochemical analysis of MLH1, MSH2, and MSH6 could predict the identified germline mutation. The observation of MSI in endometrial hyperplasia and of altered protein staining for the MMR genes supports the idea that inactivation of MMR genes is an early event in endometrial tumourigenesis. A correlation was found between the variation in the extent and level of MSI and the age of onset of carcinoma, suggesting differences in the rate of tumour progression. A high frequency of MSI in hyperplasias, found only in *MSH2* mutation carriers, might indicate a more rapid tumour progression, correlating with an earlier age of onset of carcinoma. The present study indicates that assessment of altered protein staining combined with MSI analysis of endometrial tumours might direct the mutational analysis of MMR genes.

2. INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), or Lynch syndrome, is an autosomal dominant disease characterized by an excess of colorectal cancer, endometrial cancer, and/or a variety of other cancers [1,2]. The disease is caused by a defect in the DNA mismatch repair system. Germline mutations in five genes involved in the human DNA mismatch repair (MMR) mechanism have been identified: *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6* [3]. Three of these genes, MSH2, MLH1, and MSH6, account for the MMR defect in the majority of HNPCC families. Defects in (one of) these genes lead to genetic instability characterized by expansion or contraction of simple repeat sequences (microsatellite instability, MSI). Genetic instability is responsible for a rapid accumulation of somatic mutations in different oncogenes and tumour suppressor genes which play a crucial role in tumour initiation and progression [4].

Among the extracolonic malignancies that occur in HNPCC, endometrial cancer is the most common and was recently included in the clinical criteria for HNPCC (Amsterdam II criteria)[5]. A high frequency of microsatellite instability (MSI-high phenotype) in endometrial tumours has been reported in 25-30% of sporadic endometrial carcinomas, particularly of the endometrioid subtype [6,7]. Such tumours, usually referred to as MSI-high endometrial tumours, were shown to be diploid [6], indicating an absence of chromosomal instability; this is in contrast with MSI-stable non-endometrioid endometrial carcinomas, which are generally aneuploid [8,9].

No advanced MSI analysis of HNPCC-associated endometrial carcinomas has been described yet.

Classical HNPCC is characterized by an early age of onset of colorectal carcinoma (mean age of 44 years) [4]. Recently, *MSH6* germline mutations have been identified in patients from atypical HNPCC families with a later age of onset of carcinomas and a high frequency of extracolonic malignancies, in contrast to usual HNPCC families with germline mutations in *MLH1* or *MSH2* [10,11]. In a study by our group, we observed that endometrial carcinoma was the main clinical manifestation of (atypical) HNPCC among females from families with *MSH6* germline mutations [12]. We found that the endometrial carcinomas from these families demonstrated only instability of mononucleotide repeat markers.

Previous authors have recommended MSI analysis in families with a low probability of detecting a mutation in MMR genes as a first screening test for MMR defects [13,14], but the detection of MSI in tumours does not define which one of the MMR genes is involved. A more direct assessment of the specific MMR gene involved might be possible by immunohistochemical analysis of MMR genes, as was shown for MLH1 and MSH2 in colorectal carcinomas [15].

The aims of the present investigation were, firstly, to study microsatellite instability (MSI phenotype) in a series of HNPCC-associated endometrial tumours from patients with germline mutations in different MMR genes and, secondly, to correlate MSI phenotype, mutation status, and immunohistochemical protein staining for MLH1, MSH2, and MSH6 in endometrial carcinoma and hyperplasia. It was of particular interest to study MSI and MMR protein staining in endometrial hyperplasia, to assess whether defective DNA mismatch repair by inactivation of MMR genes is an early event in the tumourigenesis of the uterine mucosa.

3. MATERIALS AND METHODS

3.1. Patient selection

We analysed in total 31 endometrial and six colorectal tumour foci in HNPCC patients from 15 families complying with the Amsterdam I criteria [16], in which a germline mutation in one of the MMR genes *MLH1*, *MSH2* or *MSH6* had been identified. For endometrial tumour foci, we investigated 23 carcinomas and eight hyperplasias. From four HNPCC patients (patient ID: 102-1, 5-12, 5-15, and 88-2), both endometrial carcinoma and associated hyperplasia were analysed.

Histological review was based on the corresponding haematoxylin and eosin (H&E)-stained tissue sections for determination of tumour cell type and grade, using the criteria of AFIP [17] and FIGO [18]. The group of 31 endometrial tumour foci consisted of 14 endometrioid adenocarcinomas (B1), two endometrioid adenocarcinomas with squamous differentiation (B2), one serous papillary adenocarcinoma (B3), one squamous cell carcinoma (B6), one mixed carcinoma (B7/B9), four endometrioid adenocarcinomas with complex hyperplasia with atypia (A4/B1), six complex hyperplasias with atypia (A4), one complex hyperplasia without atypia (A3), and one simple hyperplasia with atypia (A2) (Table 1).

According to the FIGO criteria [18], 12 carcinomas were classified as grade 1, eight carcinomas as grade 2 (patient IDs: 9796-1, 91-1, 7-96, 5-11, 867-6, 686-3, 686-4, and 22-52), and three carcinomas as grade 3 tumours (patient IDs: 686-5, 22-6, and 50176-3); low grade denotes a predominance of gland formation and high grade its relative absence. FIGO stage was assigned on the basis of histological reports and findings. Seventeen carcinomas were classified as stage IA, IB or IC; one carcinoma as stage IIA; and one carcinoma as stage IIIA.

Tumour-type	Patient ID	Histology-AFIP	MSI		Immunohistochemistry		
			Total	NCI	MLH1	MSH2	MSH6
MLH1 germline mutation							
E	102-1	A3	6/24	0/3	+	+	0
E	5-12	A4	2/40	0/4	+	+++	+
E	5-15	A4	3/25	0/3	+	+++	+++
E	9769-2	A4/B1	25/30	5/5	0	+++	++
E	9769-1	B1	22/28	5/5	++	+++	++
E	91-1	B1	11/28	3/5	+	+++	++
E	7-96	B1	22/29	3/3	+	++	+
E	102-1	B1	12/27	3/5	+	+	0
E	5-11	B1	18/28	3/4	+	++	0
E	5-12	B1	22/40	4/5	+	+++	+
E	5-15	B2	25/28	4/5	+	+++	+++
C	5-11	-	22/32	2/3	+	++	+++
C	9769-1	-	18/25	2/3	0	++	+
MSH2 germline mutation							
E	139-1	A2	20/32	4/5	+	0	0
E	88-2	A4	12/34	2/4	+	0	0
E	99-1	A4	11/34	3/5	++	0	0
E	139-1	A4	18/33	3/5	+	0	0
E	57-33	A4/B1	11/38	3/5	+++	0	0
E	77-2	B1	6/9	2/3	++	0	0
E	88-2	B1	14/26	2/3	+	0	0
E	90-1	B1	16/30	2/4	++	0	0
C	77-2	-	19/24	4/4	++	0	0
C	99-1	-	22/33	5/5	+	0	0
MSH6 germline mutation							
E	50176-5	A4	3/35	2/5	+	++	0
E	867-1	A4/B1	3/36	2/5	+++	+	0
E	867-6	B1	3/36	2/5	+++	+	0
E	22-2	B1	1/25	0/5	na	na	0
E	50176-2	B1	2/36	1/5	++	+	0
E	50176-4	B1	2/36	0/5	+++	++	0
E	686-3	B1	1/36	2/5	+++	+	0
E	686-4	B1	2/36	1/5	+++	+	0
E	686-5	B2	3/36	0/5	+++	+	0
E	22-6	B3	4/27	2/4	++	+	0
E	22-52	B6	0/31	0/4	0	++	0
E	50176-3	B7	3/36	1/5	+	+	0
R	50176-2	-	19/23	4/5	+	0	0
R	867-1	-	10/36	4/5	+	++	0

Table 1. Clinicopathological, microsatellite instability (MSI), and immunohistochemical analysis of HNPCC-associated tumours.

Tumour type: E, endometrial; C, colorectal; R, rectum; patient ID, family-patient number; clinicopathology: AFIP [17]: A2, simple hyperplasia with atypia; A3, complex hyperplasia without atypia; A4, complex hyperplasia with atypia; B1, endometrioid adenocarcinoma; B2, endometrioid adenocarcinoma with squamous differentiation; B3, serous papillary adenocarcinoma; B6, squamous cell carcinoma; B7, mixed carcinoma; microsatellite instability: total, set of 40 microsatellite markers; NCI, international set of five microsatellite markers [19]; +, instability; 0, no instability; immunohistochemistry: +++, strong; ++, moderate; +, weak; 0, no staining; na, not analysed.

3.2. DNA isolation

Genomic DNA of normal and tumour tissue was isolated from formalin-fixed, paraffin-embedded material by microdissection. In all cases except one (patient ID: 867-6), normal epithelial and tumour cells were microdissected from tissue embedded in the same paraffin block. From five cases (patient IDs: 91-1, 9769-1, 50176-2, 686-3, and 686-5), tissue was obtained by curettage, but from all other cases, including 686-5, resection material was available. Using a Chelex extraction method, DNA was isolated from five consecutive 10 µm sections, resuspended in 250 µl of PK-1 lysis buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl2, 0.45% NP40, 0.45% Tween 20, 0.1 mg/ml gelatine] containing 5% Chelex beads (Biorad, Hercules, USA) and 10 µl of proteinase K (10 mg/ml), and incubated for 12 h at 56°C. The suspension was incubated 10 min at 100°C, centrifuged, and the supernatant was carefully decanted.

3.3. Microsatellite instability analysis

We analysed 40 microsatellite markers, including five markers (two mononucleotide repeats: BAT25, BAT26; and three dinucleotide repeats: D2S123, D5S346, D17S250) recommended by the 1997 National Cancer Institute (NCI) workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition held in Bethesda, December 1997 (referred to as the NCI set) [19]. Also analysed were one mononucleotide repeat: BAT40; 16 dinucleotide (CA) n repeats: TP53, D1S158, D3S1029, D3S1611, D7S255, D8S133, NEFL, D10S197, D11S901, D11S35, D11S968, D13S175, D13S153, D17S588, D18S58, D18S61; four trinucleotide repeats: FABP2, DRPLA, D4S243, D17S1288; and nine tetranucleotide repeats: D3S1768, D3S2456, D4S1629, D8S1130, D11S1998, D13S321, D15S1232, D16S752, D17S1537. In addition, five mononucleotide repeats in the coding regions of *transforming growth factor receptor type II (TGFRII)*, *insulin-like growth factor receptor type II (IGFRII)*, and *BAX* [20-22], as well as the mismatch repair genes *MSH3* and *MSH6*, were analysed [23]. All primer sequences for microsatellite repeat markers can be attained from the Genome Database (http://www.gdb.org).

One microlitre of DNA was loaded on a polyvinyl-chloride microtitre plate (Dynatech Laboratories, Chantilly, USA). Polymerase chain reaction (PCR) was performed in a total reaction volume of 12 µl, containing 10 pmol of forward and reverse primer, 1% BSA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM MgCl2, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP, 0.2 µM fluorescent dCTP (R110 or R6G) or 0.8 µM (Tamra) (PE Applied Biosystems Inc., Foster City, USA), and 2.5 U of AmpliTaq gold polymerase (PE Applied Biosystems Inc., Foster City, USA) for 12 min at 96°C and 33 cycles of 1 min at 96°C, 2 min at 55°C, 1 min at 72°C, and a delay extension step of 7 min at 72°C in a GeneAmp 9700 thermocycler (PE Applied Biosystems Inc., Foster City, USA). 3.5 µl of loading mix (2.5 µl formamide, 0.5 µl of xylene-cyanol loading dye, 0.5 µl of Rox-500 size standard) (PE Applied Biosystems Inc., Foster City, USA) was added to 1.5 µl of sample (0.5 µl of R110, 0.5 µl of R6G, and 0.5 µl of Tamra labelled PCR product). After denaturation for 5 min at 96°C, 3.0 µl was subjected to electrophoresis on a 0.1 mm thick, 6% polyacrylamide gel containing 7 M urea and 1xTBE [0.09 M Tris- borate, 20 mM EDTA (pH 8.0)] on an ABI 377 sequencer (PE Applied Biosystems Inc., Foster City, USA) for 3 h with run profile GS-2400A and analysed with GeneScan 2.0.

Instability of a microsatellite marker is characterized by additional PCR products from the tumour DNA, compared with the electrophoresis profile of the matching normal DNA. This is facilitated by loading the differently fluorescent-

labelled PCR products from tumour and normal DNA in the same lane.

According to the recently established international criteria for MSI at the NCI workshop [19], tumours were classified as (i) tumours with instability for two or more markers (MSI-high); (ii) tumours with instability for one marker (MSI-low); and (iii) no instability (MSI-stable). Since these criteria for MSI analysis are based on studies of colorectal carcinomas and have not been tested for endometrial carcinomas, the MSI results in this report were presented based on the set of 40 markers and the NCI set (Table 1).

3.4. Immunohistochemical analysis of MLH1, MSH2, and MSH6

Immunohistochemical staining was performed on 3 µm sections of formalin-fixed, paraffin-embedded tissues using standard procedures. After antigen retrieval by boiling for 10 min in 1 mM EDTA (pH 8.0) for MLH1 and MSH2, and in 10 mM citrate buffer (pH 6.0) for MSH6, tissue sections were incubated overnight at room temperature with antibodies against human MLH1 (clone 14; Calbiogen, Cambridge, USA), dilution 1:75; human MSH2 (clone GB-12; Calbiogen, Cambridge, USA), dilution 1:75; or human MSH6 (clone 44; Transduction Laboratories/ Becton Dickinson, Hamburg, Germany), dilution 1:800. Immunoreactivity was detected using an Envision two-step peroxidase detection system (DAKO, Denmark). Staining was evaluated semi-quantitatively, using normal epithelial cells or the centres of lymphoid follicles as an internal control. Both the intensity of nuclear staining, presented in Table 1 as strong (+++), moderate (++), weak (+), or absent (0), and the percentage of tumour cells showing positive nuclear staining were scored in the area which was microdissected for MSI analysis. The latter scoring did not, however, alter the overall interpretation of the individual cases and is therefore not shown.

4. RESULTS

4.1. Clinicopathological features of endometrial carcinoma

Seventy-eight per cent (18/23) of endometrial carcinomas were of the endometrioid subtype (histology AFIP) [17]. According to the FIGO criteria [18], 12 of 23 (52%) carcinomas were grade 1 tumours, eight of 23 (35%) were grade 2 tumours and there were three grade 3 tumours. The majority of carcinomas (89%) were classified as FIGO stage I. The mean age of onset of all endometrial carcinomas was 48.7 years. A significantly earlier age of onset of endometrial carcinoma was found in patients with a germline mutation in *MSH2* than in *MLH1* mutation carriers (41.0 vs. 49.5 years, p=0.032). The mean age of onset of endometrial carcinoma in *MSH6* mutation carriers (55.5 years) was significantly later than in *MLH1* and *MSH2* mutation carriers (p=0.005 and p=0.006, respectively).

4.2. Microsatellite instability analysis

In the group of eight MLH1 mutation carriers, a MSI-high phenotype (according to the NCI criteria [19]) was observed in all endometrial carcinomas. Instability of all types of repeat markers was found by analysing the set of 40 markers (Table 1). Remarkably, in two foci of endometrial complex hyperplasia with nuclear atypia, we found instability of predominantly tri- and tetranucleotide repeat markers (i.e. FABP2, D3S2456, D8S1130, D15S1232, and D16S752). In one focus of endometrial complex hyperplasia without nuclear atypia (patient ID: 102-1), instability was found in 25% (6/24) of the markers investigated, involving all types of repeat markers (D15S1232, D8S1130, FABP2, D13S175, D18S58, and BAT40). No instability was found in the NCI set of markers in the foci of endometrial hyperplasia. Tumours were therefore classified as MSI-stable (Table 1).

Endometrial hyperplasias and carcinomas from six *MSH2* mutation carriers demonstrated an MSI-high phenotype involving all types of repeat markers. Instability was also found in two or more markers from the NCI set (Table 1).

In the group of 12 *MSH6* mutation carriers, a low frequency of instability was observed in all endometrial tumours by using the set of 40 markers. Instability was only found in the mononucleotide repeat markers (Table 1). According to the NCI criteria, four endometrial carcinomas were classified as MSI-high since BAT25 and BAT26, included in the NCI set, exhibited instability. In three endometrial carcinomas, instability was observed of only one of these two mononucleotide repeat markers, and in four endometrial carcinomas no instability was found of BAT25 or BAT26. These tumours were classified as MSI-low and MSI-stable, respectively (Table 1). One endometrial complex hyperplasia with nuclear atypia demonstrating instability of BAT25 and BAT26 was classified as MSI-high.

We also investigated the instability for five intragenic mononucleotide repeat sequences in *TGFRII*, *IGFRII*, *BAX*, *MSH6*, and *MSH3*, respectively, which frequently show instability in colorectal tumours [20-23]. Contraction of the A(10) repeat sequence (deletions) in *TGFRII* was found in all six colorectal carcinomas tested, but in none of the endometrial tumours. Contraction of the (A)8 repeat in *IGFRII* and the (G)8 repeat in *BAX* was found in respectively four and five of 23 endometrial carcinomas, but in none of the colorectal carcinomas. Interestingly, no instability was found of the (G)8 in *BAX* in an endometrial complex hyperplasia without atypia from a patient (patient ID: 102-1) with a germline mutation in *MLH1*, whereas contraction of this repeat was present in the endometrial carcinoma from the same patient. Contraction of the (C)8 in *MSH6* and the (A)8 in *MSH3* were found in respectively two and one of 12 endometrial carcinomas from *MLH1* and *MSH2* mutation carriers. As we have shown previously [12], in MSH6 mutation carriers, alterations in the (C)8 in *MSH6* were found in all endometrial and colorectal carcinomas, whereas alterations in the (A)8 in *MSH3* were only found in the colorectal carcinomas.

4.3. Immunohistochemical analysis of MLH1, MSH2, and MSH6

Loss of or markedly reduced nuclear staining for MLH1, MSH2 or MSH6, respectively, was found in all tumours from HNPCC patients (Table 1 and Figure 1). In all specimens, pre-existing glands or germinal centres staining for MLH1, MSH2, and MSH6 were retained, although we observed variations in the staining intensity, probably related to differences in the fixation standards and intervals of tissue storage.

In the majority of endometrial carcinomas and hyperplasias from *MLH1* mutation carriers a reduced or complete loss of MLH1 nuclear staining was found. In 5/11 of these tumours, both MSH2 and MSH6 staining was retained. Furthermore, in both the endometrial hyperplasia and carcinoma from one patient (patient ID: 102-1), markedly reduced staining of MSH2 and loss of MSH6 staining were also found. In three endometrial carcinomas, additional reduced (patient IDs: 5-12 and 7-96) or absent (patient ID: 5-11) MSH6 staining was found. All tumours from *MSH2* mutation carriers showed loss of MSH2 and complete loss of MSH6 staining. Conversely, in tumours from *MSH6* mutation carriers, loss of MSH6 staining and primarily reduced MSH2 staining were found. In two endometrial carcinomas and four foci of hyperplasia from *MSH2* and *MSH6* mutation carriers, reduced MLH1 staining was also observed.

It is remarkable that in an area of complex hyperplasia without atypia (patient ID: 102-1, Table 1 and Figure 1B) and in an area of simple hyperplasia without atypia (patient ID: 57-33,

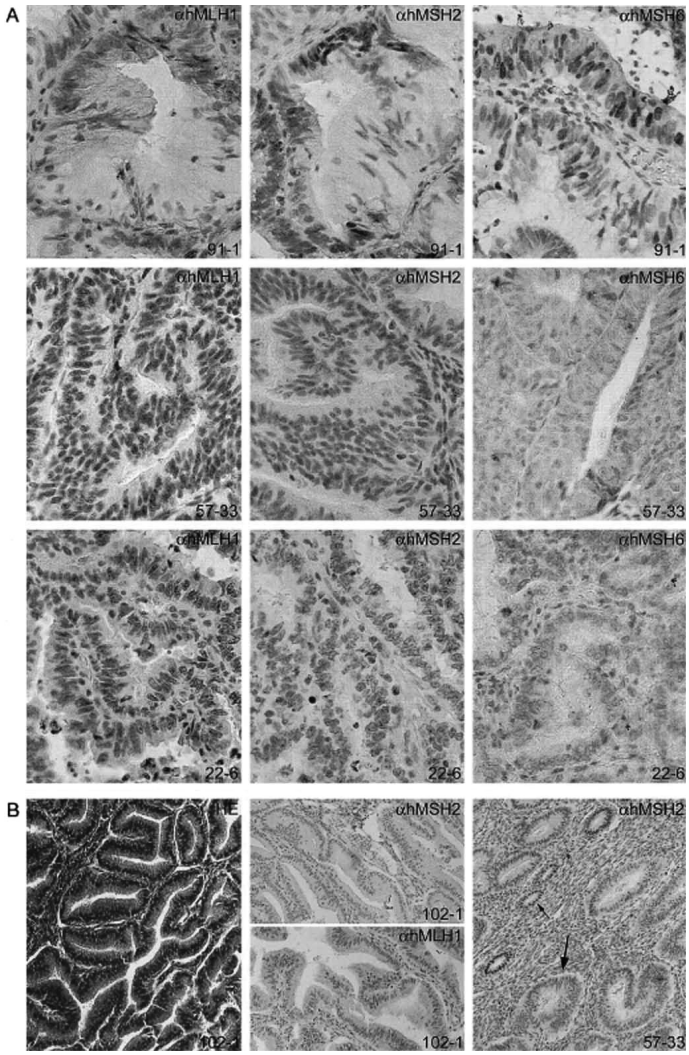

Figure 1. (A) Immunoreactivity for MLH1, MSH2, and MSH6 in three endometrial tumours from Table 1. First row: endometrial carcinoma of patient 91-1 (MLH1 mutation carrier) stained with anti-human MLH1, MSH2, and MSH6 antibodies, respectively. MLH1 nuclear staining is reduced. MSH2 and MSH6 staining is retained. Second row: endometrial carcinoma of patient 57-33 (MSH2 mutation carrier) stained with anti-human MLH1, MSH2, and MSH6 antibodies, respectively. Nuclear staining for both MSH2 and MSH6 is absent (abrogation MutS/). MLH1 staining is retained. Third row: endometrial carcinoma from patient 22-6 (MSH6 mutation carrier) stained with anti-human MLH1, MSH2, and MSH6 antibodies, respectively. MLH1 staining is retained. Nuclear staining for MSH6 is absent. MSH2 staining is reduced (abrogation MutS). (B) Areas of complex and simple endometrial hyperplasia. Left: H&E staining of the complex hyperplasia without atypia from patient 102-1 (MLH1 mutation carrier, Table 1). Middle: same tumour area stained with anti-human MSH2 and MLH1 antibodies, respectively. With both antibodies, nuclear staining is markedly reduced (MSH6 staining was absent, not shown). Right: area of simple hyperplasia without atypia from patient 57-33 (MSH2 mutation carrier). In hyperplastic glands, MSH2 staining (large arrow) is absent, whereas in normal glands MSH2 nuclear staining (small arrow) is retained.

Figure 1B) diminished or absent nuclear staining of MLH1 or MSH2, respectively, can already be observed.

5. DISCUSSION

In the present study we performed MSI and immunohistochemical analysis in endometrial carcinomas and hyperplasias from patients with identified gene mutations in *MLH1*, *MSH2* or *MSH6*. All endometrial carcinomas from carriers of *MLH1* and *MSH2* germline mutations showed, according to the NCI citeria [19], an MSI-high phenotype involving all types of repeat markers, while endometrial carcinomas from *MSH6* mutation carriers exhibited an MSI-high phenotype in only 36% (4 out of 11) of the tumours. An overall low frequency of MSI was found by analysing the set of 40 markers, but in all *MSH6*-associated tumours, instability was observed only of the mononucleotide repeat markers. Interestingly, an MSI-high phenotype involving all types of repeat markers was found in endometrial hyperplasias from MSH2 mutation carriers, but not in hyperplasias from MLH1 mutation carriers exhibiting an MSI-stable phenotype. In these hyperplasias, extensive analysis with the set of 40 markers detected instability predominantly of tri- and tetranucleotide repeat markers. In conclusion, in our study we found a mutated MMR gene-specific phenotype of MSI in endometrial hyperplasia from HNPCC patients.

The immunohistochemical analysis using antibodies directed against the MMR gene proteins MSH2 and MSH6 showed a complete loss of staining for these proteins in both endometrial carcinomas and hyperplasias from HNPCC patients, concordant with identified germline mutation in these genes. Reduced staining for MLH1 was found in endometrial carcinomas and hyperplasias from *MLH1* mutation carriers. In tumours from *MSH2* mutation carriers, absent staining for the MSH6 protein was also observed, while conversely, in tumours from *MSH6* mutation carriers, reduced staining for MSH2 was found. These reduced nuclear staining patterns are most likely the result of abrogation of the MutS complex [24,25]. Residual MSH2 staining in tumour cells might be due to MSH2 protein within the MutS complex. The observation of MSI in endometrial hyperplasia, and altered protein staining for MLH1, MSH2, and MSH6, respectively, concordant with identified germline mutation in one of these MMR genes, supports the hypothesis that inactivation of MMR genes is an early event in endometrial tumourigenesis. The finding of MSI and altered MMR protein staining in one endometrial complex hyperplasia without atypia is remarkable. Endometrial hyperplasia without atypia is a manifestation of unopposed oestrogenic stimulation, but by itself is not thought to be a precursor of endometrial carcinoma.

The observation of an MSI-high phenotype in both endometrial hyperplasias and carcinomas from *MSH2* mutation carriers, the MSI-high phenotype in carcinomas but not in hyperplasias from *MLH1* mutation carriers, and the predominantly MSI-low or -stable phenotype in carcinomas from *MSH6* mutation carriers, may suggest differences in tumour progression. The variation in the extent and level of MSI may account for the differences in the age of onset of endometrial cancer between carriers of germline mutations in different MMR genes. The series of HNPCC-associated endometrial carcinomas in the present study has a relatively early age of onset (mean 48.7 years) compared with sporadic cases (mean 59 years) [26]. Interestingly, we observed a significant difference in the age of onset of endometrial carcinoma between patients with a germline mutation in the MMR genes *MLH1*, *MSH2*, and *MSH6* (49.5, 41.0, and

55.5 years, respectively). The high frequency of MSI in endometrial hyperplasias found only in *MSH2* mutation carriers, correlated with an early age of onset of carcinoma, might indicate rapid tumour progression. The predominantly MSI-low or stable phenotypes in carcinomas from *MSH6* mutation carriers correlate with a later age of onset of carcinoma, indicating a slower tumour progression rate.

Previous studies have evaluated genes containing intragenic mononucleotide repeats. A high frequency of instability of *BAX*, *TGFRII*, *IGFIIR*, *MSH3*, and *MSH6* has been reported in colorectal carcinomas [20-23]. We identified instability of *BAX*, *IGFRII*, and *TGFRII* in respectively 22%, 17%, and 0% of endometrial carcinomas, similar to previous studies of MSI in sporadic endometrial carcinomas [6,7,27-30]. On the other hand, in all endometrial carcinomas from *MSH6* mutation carriers, there was instability of the (C)8 repeat in *MSH6* [12]. In contrast, only 17% of carcinomas from *MLH1* and *MSH2* mutation carriers demonstrate instability of *MSH6*. In sporadic endometrial carcinomas, instability of *MSH6* was reported in 7% of tumours [7]. The high frequency of instability in our series of HNPCC-associated endometrial tumours might indicate that *MSH6* represents a preferential target for somatic mutations in *MSH6* mutation carriers, which is in agreement with earlier observations [10-12]. Although inactivation of *MSH6* might be important for tumour progression in *MSH6* mutation carriers, other molecular targets of MSI in endometrial tumourigenesis remain to be elucidated.

The search for MMR gene mutations is time-consuming and thus expensive. Wijnen et al. described that if the probability of detecting a mutation in MMR genes based on clinical features in HNPCC families is low, MSI analysis should be considered as an initial screening test for MMR defects [13]. The present study has shown that it is possible to predict which MMR gene is mutated by using MSI and immunohistochemical analysis of MLH1, MSH2 and MSH6 in endometrial tumours. In 29 out of 31 (94%) endometrial tumour foci, both carcinoma and hyperplasia, combined MSI and immunohistochemical analysis could predict the identified germline mutation. The distinction between *MSH2* and *MSH6* defects, however, is very subtle; instability of only mononucleotide markers indicates a *MSH6* defect.

In endometrial carcinomas, MSI analysis alone could not predict the identified germline mutations in the MMR genes *MLH1* and *MSH2*, since a high frequency of instability is found in all types of microsatellite markers in both groups. However, a focus of hyperplasia with instability in all types of microsatellite markers (MSI-high) might indicate an *MSH2* defect. Selective instability in predominantly mononucleotide repeat markers in both endometrial carcinomas and hyperplasias from *MSH6* mutation carriers can predict *MSH6* germline mutations in 8/12 tumours, using the NCI set, and in 11/12, using the extended marker set.

Only in 3/11 endometrial tumour foci of *MLH1* mutation carriers could immunohistochemical analysis alone predict the germline mutation in *MLH1*. In the group of *MSH2* and *MSH6* in 8/8 and 9/12 tumours, respectively, immunohistochemistry could predict a potential *MSH2* or *MSH6* mutation. However, although the immunohistochemical data seem promising, we do not favour the use of immunohistochemistry alone to direct mutation analysis. Distinction between *MSH2* and *MSH6* defects by immunohistochemistry is very subtle and the staining pattern predicting an *MLH1* defect is unreliable in our hands. In families with two or more endometrial cancers, with an endometrial cancer patient diagnosed at an unusually young age, or with colorectal and endometrial cancer,

in which colorectal cancer tissue is not available, we recommend MSI analysis and immunohistochemistry as a first investigative step.

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