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Defective mismatch-repair in patients with multiple primary tumours including colorectal cancer

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Abstract

Individuals with an inherited predisposition to cancer development are at an increased risk of developing multiple tumours. Hereditary non-polyposis colorectal cancer (HNPCC) is one of the most common hereditary cancer syndromes and is estimated to account for approximately 2% of colorectal cancers. However, HNPCC individuals are at an increased risk of developing other tumour types such as cancers of the endometrium, urothelium and small intestine. We have utilised a population-based regional cancer registry to identify all patients with double primary colorectal cancers and at least one additional malignancy and characterised the tumour spectrum in this patient group. We subsequently selected those 47 individuals who had developed at least four malignancies, including two colorectal cancers, for studies of the tumour characteristics associated with HNPCC. In total, these individuals developed 209 tumours, 156 of which were successfully retrieved. Microsatellite instability (MSI), a phenomenon caused by defective mismatch-repair (MMR), was identified in 63/154 (41%) evaluable tumours with a MSI-high pattern in 59 and a MSI-low pattern in four tumours. All tumours were immunohistochemically stained for the MMR proteins MLH1 and MSH2, with loss of expression in 55/63 (87%) MSI tumours and in 2/89 (2%) microsatellite stable (MSS) tumours. This loss affected MLH1 in 24 tumours and MSH2 in 33 tumours. A concordant loss of expression for the same MMR protein in several tumours from the same individual, a pattern that strongly suggests an underlying germline MMR gene mutation, was found in 17/45 (38%) patients and affected MLH1 in 8 patients and MSH2 in 9 patients. We conclude that the development of multiple primary tumours, including synchronous or metachronous colorectal cancers, is associated with an increased frequency of MSI and loss of immunohistochemical expression of MLH1 and MSH2.

Keywords: Multiple primary tumours; HNPCC; MSI; MMR; Immunohistochemistry; MSH2; MLH1

1. Introduction

Less than 1% of cancer patients develop more than two primary tumours and multiple primary tumours in an individual has been recognised as an indicator of a genetic predisposition to cancer [1]. One of the most common inherited cancer syndromes, hereditary non-polyposis colorectal cancer (HNPCC), has been estimated to affect approximately 1/1000 individuals and to account for 1–2% of colorectal cancer [2–4]. HNPCC patients are at an increased risk for several cancer types, with the greatest life-time risks for colorectal cancer (80% risk for mutation carriers) and endometrial cancer (40–60% risk) and increased risks also for cancer of the urothelium, small intestine, ovary, ventricle, hepatobiliary tract and skin [5,6]. The increased risk of several cancer types implies that the development of multiple primary cancers is common in HNPCC families and second primary malignancies have been estimated to occur in approximately 40% of the patients [6]. HNPCC is caused by a germline mutation in a mismatch-repair (MMR) gene, most commonly affecting *MLH1, MSH2*, and *MSH6* [7]. Clinical criteria, the Amsterdam II criteria, for the classification of HNPCC have been established and

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consider cancers of the colorectum, endometrium, urothelium and the small bowel as being associated with HNPCC and request that two successive generations should be affected with at least one individual diagnosed before the age of 50 years [5]. In the clinical setting, a family history of cancer is considered the most useful indicator of HNPCC. However, reduced penetrance and occurrence of *de novo* mutations should be taken into account when performing genetic councelling [8]. Individuals with a weak family history of cancer who have themselves developed multiple primary HNPCC-associated tumours are not included in the Amsterdam II criteria and may thus be excluded from genetic testing.

Microsatellite instability (MSI) has been recognised as a hallmark of HNPCC and is found in >95% of HNPCC tumours [9], but MSI is also found in a subset of several types of sporadic cancer with the highest frequencies, 15–20%, in colorectal cancer, endometrial cancer and gastric cancer [10–12]. Germline mutations of *MLH1* and *MSH2* occur at approximately equal frequencies in HNPCC, whereas virtually all sporadic MSI-high cancers develop due to epigenetic silencing of the *MLH1* promoter through somatic hypermethylation [11,13–15]. The MMR proteins are ubiquitously expressed in proliferating tissue and loss of expression for a MMR protein in the tumour tissue indicates an underlying MMR defect. A good correlation between MSI and loss of MMR protein expression has been demonstrated and immunohistochemical staining may thus be used to pinpoint the mutated/hypermethylated MMR gene [15–17].

We have utilised a regional, population-based cancer registry to identify all individuals who have developed synchrononous or metachronous colorectal cancer and at least one additional malignancy. The tumour spectrum in this group of patients was characterised and in order to clarify the contribution of defective MMR to the development of multiple primary tumours in an individual, patients who had developed four or more primary cancers (at least two of which were colorectal adenocarcinomas) were selected for studies of MSI and immunohistochemical MMR protein expression in the tumour tissue.

2. Patients and methods

Ethical approval for the study was obtained from the ethics committee at Lund University. The patients were identified through the population-based cancer registry of the southern Swedish health care region, which currently has approximately 1.5 million inhabitants. The registry was established in 1958 and because of a mandatory cancer registration for clinicians, as well as for pathologists, the registry is estimated to contain 98% of all cancers diagnosed. All malignant diagnoses with the addition of, for example, meningiomas and certain other specified benign neoplasms are included in the registry. All patients diagnosed with double primary colorectal cancer, either synchronous or metachronous, with at least one other primary malignancy during the time period 1958–2000 were identified. Double primary colorectal cancers and at least one additional malignancy had developed in 264 patients.

We selected 47 patients who had developed at least four malignancies (two of which were colorectal cancers and which could include a meningioma) for studies of tumour biology compatible with defective MMR. The material included 30 men and 17 women with a median age at first diagnosis of 65 years (range 26–91 years). These patients had developed 207 tumours, which included 133 colorectal cancers, 20 prostate cancers, 13 urothelial cancers, 5 skin cancers, 4 melanomas, 5 endometrial cancers, 5 breast cancers, 2 lung cancers, 3 gastric cancers, 2 cancers of the small intestine, 2 lymphomas, 2 chronic leukaemias, 2 laryngeal cancers, 2 meningiomas, 1 sarcoma, 1 thyroid cancer, 1 primary liver cancer, 1 ovarian cancer, 1 cervical cancer, 1 cancer of the vagina, 1 mesothelioma, 1 parathyroid cancer and 1 cancer of the eye. We successfully retrieved 156 tumours which included 114 colorectal cancers, 6 prostate cancers, 10 urothelial cancers, 4 skin cancers, 3 melanomas, 2 endometrial cancers, 4 breast cancers, 3 gastric cancers, 2 cancers of the small intestine, 1 lung cancer, 1 mesothelioma, 1 ovarian cancer, 1 sarcoma, 1 meningioma, 1 thyroid cancer, 1 cervical cancer and 1 cancer of the vagina. Evaluable data on MSI and immunohistochemical MMR protein expression was obtained from 154 tumours. All diagnoses were checked by retrieval of the original histopathological reports and a 4-μm section from all tumours was stained with Haematoxylin and Erythrosin for routine morphology to verify that representative, non-necrotic tumour tissue was present in the tumour block.

2.1. MSI analysis

DNA was extracted from three 10-μm sections of formalin-fixed, paraffin-embedded tumour tissue. The sections were incubated at 65 °C with proteinase K (50mM Tris, pH 8.4, 1mM ethylene diamine tetra acetic acid (EDTA), 0.5% (v/v) Tween-20, 200 μg/ml proteinase K) for at least 2 h, followed by 10 min of boiling for enzyme inactivation. The samples were then centrifuged for 5 min and the aqueous phase was transferred to a new tube. We primarily analysed the MSI markers BAT25 (an intronic $(T)_{25}$ sequence in the *KIT* gene), BAT26 (an intronic $(A)_{26}$ sequence in the *MSH2* gene) and BAT40 (an intronic (T)40 tract in the *3-β-hydroxysteroid dehydrogenase* gene) and as additional markers we used the dinucleotide markers BAT34 (a $(T)_3C(T)_6C(T)_{17}C(T)_5C(T)_3$ tract in a 3′ non-translated

part of the *TP53* gene) and D5S346 (in the *APC* gene). The markers BAT34C4, BAT26 and BAT40 are quasi-monomorphic with allelic size variations rarely exceeding 2 bp. The marker BAT40 rarely exhibits large size variations (−6 to −16 bp, although a −16 bp polymorphism has been described and was observed in two of our patients) [18]. Thus, these markers yield MSI patterns that can generally be interpreted without the need for matching normal tissue from the same patient. All markers used are among those recommended in the National Cancer Institute (NCI) reference panel for MSI analysis and have been shown to assess MSI with high accuracy [19]. In order to classify a tumour as MSS, data from at least three of the markers were required and all markers had to be negative. A tumour was classified as MSI-high if at least two of the markers were instable. For MSI-low tumours, an additional two markers were studied and tumours with instability in only 1/5 markers were classified as MSI-low. The sequences for the primers used were: BAT25: 5′-TCGCCTCCAAGAATGTAAGT-3′ (forward) and 5′-TCTGCATTTTAACTATGGCTC-3′ (reverse), BAT26: 5′-TGACTACTTTTGACTTCAGCC-3′ (forward) and 5′-AACCATTCAACATTTTTAACCC-3′ (reverse), BAT 40: 5′-ACAACCCTGCTTTTGTTCCT-3′ (forward) and 5′-GTAGAGCAAGACCACCTTG-3′ (reverse), BAT34C4: 5′-ACCCTGGAGGATTTCAT CTC-3′ (forward) and 5′-AACAAAGCGAGACCC AGTCT-3′ (reverse) and D5S346: 5′-ACTCACTC TAGTGATAAATCG-3′ (forward) and 5′-AGCAGA TAAGACAGTATTACTAGTT-3′ (reverse). The markers were fluorescence labelled as follows: BAT25 with Tet (green), BAT26 with Fam (blue), BAT40 with Hex (yellow), BAT34C4 with Fam (blue) and D5S346 with Hex (yellow). The polymerase chain reaction (PCR) amplifications were performed using AmpliTaq Gold DNA Polymerase from Applied Biosystems /Roche (Foster City, CA, USA). The PCR reactions were performed with an initial denaturation at 94 °C for 7 min, 10 cycles of denaturation at 94 °C for 15 s, annealing at 45 or 50 °C for 15 s and extension at 72 °C for 15 s, followed by 23 cycles with denaturation at 89 °C for 15 s, annealing at 45 or 50 °C for 15 s and extension at 72 °C for 15 s. Finally, the PCR products were subjected to 7 min of elongation at 72 °C, followed by a cooling step of 4 °C. An annealing temperature of 45 °C was used for BAT25, and 50 °C for BAT26, BAT40, BAT34C4 and D5S346. To verify the presence of the correct PCR product, these were subjected to electrophoresis in a 7.5% (w/v) acrylamide gel and stained with ethidium bromide. For the MSI analysis, the PCR products were combined with Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and Gene Scan™ size standard (Applied Biosystems), denatured at 95 °C for 2 min, chilled on ice and separated in Performance Optimized Polymer-4 (POP-4™, Applied Biosystems) on a ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

2.2. Immunohistochemistry

The paraffin-embedded tumour blocks were cut in 4-μm sections and mounted on DAKO ChemMate Capillary Gap Microscope Slides (DAKO A/S, Denmark). The slides were dried over night at room temperature followed by incubation at 60 °C for 1–2 h. For deparaffinisation Xylol was used, and for rehydration, alcohol in descending concentrations was used. Antigen retrieval was achieved by microwave treatment in 1 mM EDTA solution, pH 9.0, using 900W for 8 min and 350 W for 15 min, after which the slides were allowed to cool in EDTA solution for 20 min at room temperature. The immunohistochemical staining was performed in an automated immunostainer (TechMate 500 Plus, DAKO) according to the manufacturer's instructions. In brief, the procedure included incubation at room temperature for 25 min with monoclonal mouse IgG antibodies MLH1 (clone G168-15, dilution 1:100, PharMingen, San Diego, CA, USA) and MSH2 (clone FE-11, dilution 1:100. Oncogene Research Products, Boston, MA, USA) followed by incubation with biotinylated anti-mouse antibody (DAKO) for 25 min. Endogenous peroxidase activity was blocked through incubation in 3% (v/v) H_2O_2 for 3×2.5 min, followed by incubation with streptavidin-horseradish peroxidase for 25 min. Finally, the slides were treated with diaminobenzidine, counterstained with haematoxylin, dehydrated in ascending concentrations of alcohol and mounted. Tris-buffered saline, pH 7.6, and Triton-X-100 were used to rinse the slides between each step. Bovine serum albumin was added to the buffers before and after the antibody binding steps in order to block non-specific protein binding. The slides were evaluated independently by two of the authors without knowledge of the MSI results. As negative controls, we utilised tumours with mutations in *MLH1*/*MSH2* and slides without primary antibody treatment. Internal positive control staining, i.e. retained expression in the tumour stroma, in tumour infiltrating lymphocytes, and/or in surrounding normal tissue, was required in order to interpret the results. Loss of expression of the respective MMR protein was defined as complete absence of nuclear staining in the tumour cells.

3. Results

In total, the 264 patients with at least three tumours (including two colorectal cancers) developed 792 tumours. Synchronous colorectal cancer occurred in 135 patients with a mean age of 72 years at the first diagnosis, metachronous colorectal cancer occurred in 116

patients with a mean age of 67 years at the first diagnosis and 13 patients were diagnosed with both synchronous and metachronous colorectal cancers at a mean age of 73 years at the first diagnosis. Apart from colorectal cancer, prostate cancer and skin cancer were the two most common tumour types, followed by urothelial cancer, breast cancer, endometrial and ovarian cancers (Fig. 1).

3.1. MSI analysis

In total 156 tumours from 45 of the 47 patients were successfully retrieved and were found on re-evaluation to contain acceptable amounts of good-quality tumour tissue. MSI analysis classified 59 tumours as MSI-high (50/114 colorectal cancers, 1/2 of endometrial cancers, 4/9 of urothelial cancers and 3/3 of gastric cancers and in 1/2 cancers of the small intestine), four tumours as MSI-low (four colorectal cancers) and 91 tumours as MSS, whereas MSI was not possible to evaluate due to small amounts of tumour tissue or lack of PCR products in two tumours (Table 1). MSI was not detected in any cancers of the prostate, breast, lung, thyroid or in melanomas, a meningioma or a sarcoma. Among the MSI-high tumours, 45/59 tumours were instable for all markers analysed. The 4 MSI-low tumours displayed instability of BAT40 only in 3 tumours and of BAT26 only in one tumour.

3.2. MMR protein expression

MMR protein immunohistochemistry of the 156 tumours revealed loss of expression of MLH1 in 24 tumours, loss of MSH2 in 33 tumours (Fig. 2) and retained expression of both antibodies in 93 tumours, whereas the immunohistochemical staining failed in one MSS tumours and 1 MSI-high tumour showed adequate staining for MSH2 alone (Table 1). Of the MSI-high tumours 52/59 (88%) evaluable tumours showed loss of expression for at least one the MMR proteins, whereas loss of expression was found in three MSI-low tumours (Table 1). A concordant loss of expression of the same MMR protein in at least two tumours from the same patient was found in 17/45 patients and affected MLH1 in 8 patients and MSH2 in 9 patients (Table 2). Occasional MSI-positive tumours were found in 5 patients, in 3 of whom loss of MLH1 expression was found in the

Table 1
Summary of MSI and MMR expression data

MMR protein expression	MSI status[b]		
	MSI-high *n* (%)	MSI-low *n* (%)	MSS[a] *n* (%)
MLH1+/MSH2+[b]	7/59 (12)	1/4 (25)	87/89 (98)
MLH1−/MSH2+	23/59 (39)	1/4 (25)	0/89 (0)
MSH2−/MLH1+	29/59 (49)	2/4 (50)	2/89 (2)

MSI, microsatellite instability; MMR, mismatch repair; MSS, microsatellite stable.

[a] Two MSS tumours were not evaluable for MMR protein expression.

[b] One MSS tumour and one MHI-high tumour showed adequate staining for MSH2 only, which was expressed in both cases.

[c] Two tumours were not evaluable for MSI, but expressed both MLH1 and MSH2.

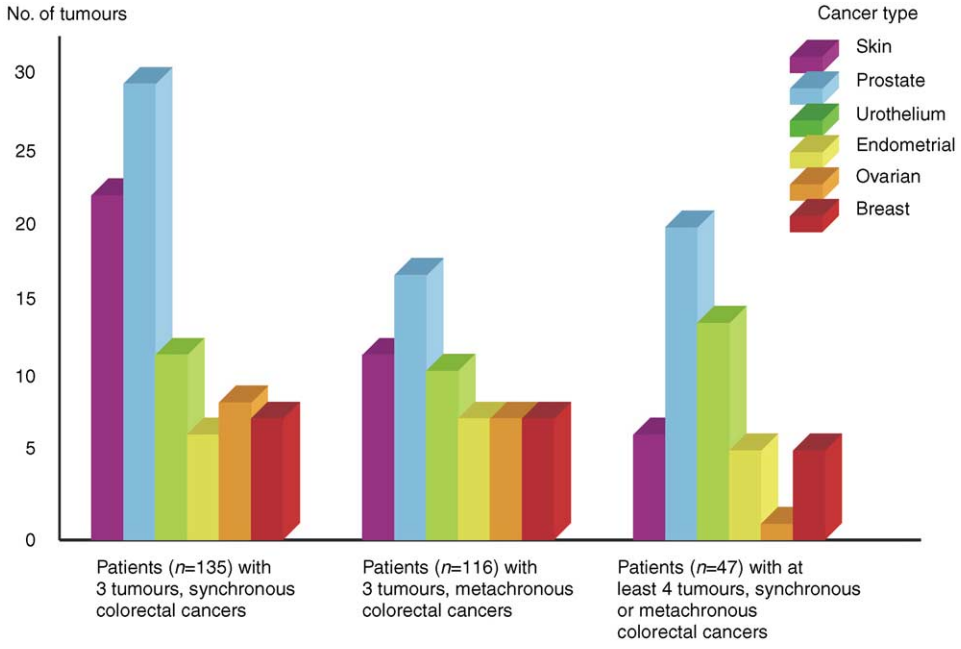

Fig. 1. Distribution of the 6 most common tumour types in patients with multiple tumours.

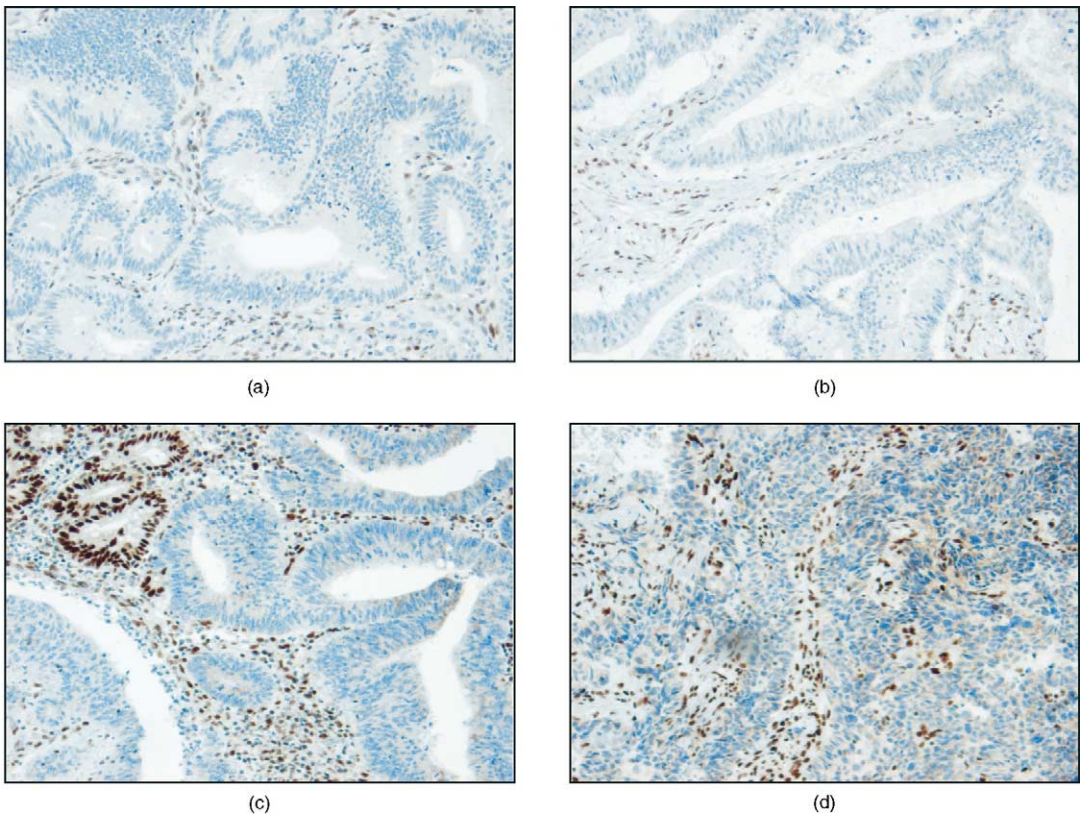

Fig. 2. Loss of immunohistochemical expression of MSH2 in four tumours from the same individual (n7): (a) colon cancer at age 35 years; (b) colon cancer at age 46 years; (c) colon cancer at age 46 years and (d) gastric cancer at age 53 years. Loss of staining for MSH2 is seen in the tumour tissue, whereas retained expression is seen in the tumour stroma.

MSI tumour (Table 3). Retained expression of both MMR proteins was found in 7/63 (11%) MSI tumours. Of the 89 evaluable MSS tumours, 87/89 (98%) showed normal MMR protein expression and 2/89 (2%), both urothelial cancers, showed loss of MSH2 protein expression.

All cases with MSI and/or loss of MMR protein expression in at least one tumour are shown in Tables 2 and 3.

4. Discussion

Development of multiple tumours in a patient has been recognised as an indicator of hereditary cancer, but most studies are limited to multiple tumours of the same histopathological type or to case reports of patients with, for example, HNPCC or Li–Fraumeni syndrome. Studies of *BRCA1* and *BRCA2* mutations in breast cancer families have shown a higher frequency of mutations in families containing individuals with breast cancer and a second primary non-breast cancer than in breast cancer-only families [20]. Colorectal cancer patients are also at an increased risk of developing cancers of the endometrium, urothelium, small intestine, ovary, ventricle, i.e. tumour types associated with HNPCC [5,6,20–25]. The cumulative incidence of a second primary colorectal cancer has been estimated to be 1.5% at 5 years, with a higher risk for patients who developed their first colorectal cancer at a young age [24,25].

Furthermore, patients with multiple colorectal cancers more often have a family history of colorectal tumours as well as of extracolonic malignancies [21–24]. In the national Swedish cancer registry, 91% of the patients developed one malignancy, 8% had two, and 1% of the patients had more than two malignancies of any histopathological type. Regarding colorectal cancer only, 97.6% of the patients developed one colorectal tumour, 2.3% developed two colorectal cancers (synchronous or metachronous) and approximately 0.1% of the patients three or more colorectal cancers [1]. We have utilised a regional, population-based cancer registry to identify patients with multiple primary tumours, including at least two colorectal cancers. Among the 264 patients who developed three malignancies including synchronous or metachronous colorectal cancers, various tumour types occurred, with the most frequently observed tumour types being cancer of the prostate, skin and urothelium (Fig. 1). Whether the patient had developed synchronous or metachronous colorectal

Table 2
Cases with MSI and/or MMR protein loss suggesting HNPCC

Case/sex	Tumour type	Age (years)	MSI	MSH2	MLH1
n1/f	Endometrial	40	H	–	+
	Colon	44	H	–	+
	Rectal	70	L	–	+
	Small bowell	70	H	–	+
	Colon	72	H	–	+
n3/f	Endometrial	46	NI	NI	NI
	Colon	61	H	–	+
	Colon	61	MSS	+	+
	Colon	69	H	–	+
	Rectal	71	H	–	+
n4/m	Rectal	40	H	–	+
	Urothelial	45	H	–	+
	Colon	56	H	–	+
	Colon	56	H	–	+
n6/m	Lymphoma	26	NI	NI	NI
	Colon	49	H	–	+
	Colon	49	H	–	+
	Sarcoma	49	MSS	+	+
n13/f	Colon	61	H	–	+
	Rectum	62	H	–	+
	Urothelial	65	H	–	+
	Urothelial	82	H	–	+
n32/m	Rectal	67	H	–	+
	Urothelial[a]	68	MSS	–	+
	Gastric	69	H	–	+
	Colon	71	H	–	+
	Prostate	71	NI	NI	NI
n35/m	Gastric	52	H	–	+
	Colon	52	H	–	+
	Colon	53	L	–	+
	Colon	54	H	–	+
	Colon	62	L	–	+
	Prostate	74	NI	NI	NI
n37/f	Urothelial	56	MSS	–	+
	Colon	59	H	–	+
	Meningioma	60	MSS	+	+
	Colon	62	H	–	+
n43/f	Endometrial	49	IC	IC	IC
	Rectal	65	H	–	+
	Colon	76	H	–	+
	Colon	76	H	–	+
n7/f	Colon	35	H	+	–
	Colon	46	H	+	–
	Colon	46	H	+	–
	Gastric	53	H	+	–
n23/m	Prostate	73	NI	NI	NI
	Colon	79	L	+	–
	Colon	79	H	+	–
	Melanoma	80	MSS	IC	IC
n30/f	Meningioma	59	IC	IC	IC
	Colon	73	H	+	–
	Rectal	73	H	+	–
	Colon	75	H	+	–
n41/m	Colon	46	NI	NI	NI
	Lung	79	NI	NI	NI
	Colon	79	H	+	–
	Colon	79	H	+	–
	Colon	79	MSS	+	+
n44/m	Colon	67	H	+	–
	Colon	75	H	+	–
	Colon	76	H	+	–
	Colon	76	H	+	–
n45/f	Colon	69	MSS	+	+
	Colon	69	H	+	–
	Colon	73	H	+	–
	Breast	74	MSS	+	+
n46/f	Cervical	58	MSS	+	+
	Colon	81	H	+	–
	Colon	81	H	+	–
	Vaginal	90	MSS	+	+
n47/m	Colon	80	H	+	–
	Colon	82	H	+	–
	Skin-CIS	85	MSS	+	+
	Skin	85	MSS	+	+
	Skin-CIS	85	MSS	+	+
n2/f	Rectal	50	H	+	+
	Colon	69	NI	NI	NI
	Colon	71	H	+	IC
	Colon	71	H	+	+
n5/f	Endometrial	45	NI	NI	NI
	Colon	48	L	+	+
	Rectal	48	H	+	+
	Breast	52	NI	NI	NI
	Colon	55	H	+	+
	Colon	56	MSS	+	+

MSI, microsatellite instability; MMR, mismatch-repair; HNPCC, hereditary non-polyposis colorectal cancer; f, female; m, male; CIS, carcinoma *in situ*; H, MSI-high; L, MSI-low; MSS, microsatellite stable; NI, not investigated; IC, inconclusive.

[a] Less than 20% tumour tissue in sample.

cancers did not seem to influence the type of additional primary tumours in the individual (Fig. 1). Studies of multiple colorectal cancers (synchronous and metachronous) occurring in the same individual have demonstrated MSI frequencies between 20 and 90% [26–31]. In one of these studies, sporadic tumours and HNPCC tumours were separately studied and demonstrated MSI frequencies of 17 and 85%, respectively [30]. Studies of women with double primary endometrial and colorectal cancers have identified MSI and germline HNPCC-causing mutation at increased frequencies [32,33]. The present study of MSI and immunohistochemical expression of MLH1 and MSH2 in 156 tumours from 45 patients with at least four primary tumours revealed MSI in 40% of all tumours, including cancers of the colorectum, ventricle, urothelium, endometrium and small intestine, all of which represent tumour types associated with a defective MMR. Of the MSI tumours, 94% were classified as MSI-high and 6%

Table 3
Tumours with MSI and/or MMR expression suggesting somatic inactivation

Case/sex	Cancer type	Age (years)	MSI	MSH2	MLH1
n10/m	Prostate	91	MSS	+	+
	Colon	93	MSS	+	+
	Colon	95	H	+	–
	Colon	95	MSS	+	+
n15/m	Urothelial	77	MSS	+	+
	Colon	77	H	+	–
	Colon	77	MSS	+	+
	Prostate	81	MSS	+	+
n26/f	Urothelial	70	NI	NI	NI
	Colon	73	MSS	+	+
	Colon	73	NI	NI	NI
	Colon	83	H	+	–
n28/m	Colon	60	IC	+	+
	Prostate	72	MSS	+	+
	Colon	81	H	+	+
	Colon	81	NI	NI	NI
	Colon	81	NI	NI	NI
n29/m	Colon	68	NI	NI	NI
	Rectum	68	NI	NI	NI
	Lung	74	MSS	+	+
	Urothelial	75	H	+	+

MSI, microsatellite instability; MMR, mismatch-repair; f, female; m, male; H, MSI-high; L, MSI-low; MSS, microsatellite stable; NI, not investigated.

as MSI-low. A low level of MSI, usually involving mononucleotide markers, may be associated with mutations in *MSH6*, whereas sporadic MSI-low tumours rarely show instability of the markers BAT25, BAT26 and BAT40 [2,34].

A good correlation between MSI and immunohistochemical loss of MMR protein expression has been demonstrated [16]. Loss of immunohistochemical expression of MSH2 is almost exclusively caused by an underlying germline mutation [15–17]. In contrast, loss of MLH1 expression might either reflect a germline mutation or a somatic inactivation of *MLH1*, which in the majority of cases is caused by epigenetic silencing through promoter hypermethylation [11,15]. In the present study, the immunohistochemical staining of MLH1 and MSH2 revealed loss of expression for at least one of the proteins in 37% of all tumours (this is (24 + 33)/154 evaluable tumours) (Table 1, Fig. 2). When the MSI findings were correlated with the results of the MMR protein expression, loss of expression occurred in 88% of the MSI-high tumours (and affected MLH1 in 31 tumours and MSH2 in 33 tumours) and three MSI-low tumours showed loss of expression (that affected MLH1 in one tumour and MSH2 in two tumours), whereas retained expression of both MMR proteins was found in 98% of the evaluable MSS tumours (Table 1). A discordant MSI/MMR protein expression status occurred in 2% of the MSS tumours that showed loss of expression for one of the MMR proteins and in the 13% of the MSI tumours that showed retained expression for both MMR proteins. A MSS phenotype with immunohistochemical loss of MSH2 occurred in two tumours, both of which were urothelial cancers (Table 2, n32 and n37). Since the sensitivity of MSI is unknown in this tumour type, these cases may represent false-negative MSI tumours which is likely since the other HNPCC-associated tumours in these patients were generally MSI-high. In six immunohistochemically evaluable MSI tumours from 4 individuals, retained expression for MLH1 and MSH2 was found (Table 2, n2 and n5; Table 3, n28 and n29). Such an MSI/MMR-expression pattern has been reported in a subset of colorectal cancers and the genetic background to this finding is unknown [16]. Some of these cases may still represent HNPCC, in which case the nature of the secondary somatic mutation could perhaps influence the MMR protein expression, or be caused by mutations in other MMR genes, such as *MSH6*, which was not investigated in this study, but is estimated to account for approximately 5–10% of HNPCC [7].

Multiple MSI tumours and/or loss of expression affecting the same MMR protein in at least two tumours from the same patient was found in 19/45 evaluable cases (Table 2). These 19 patients with suspected HNPCC developed the first tumour at mean age of 54 years (range 26–74 years) compared with a mean age of 72 years among the remaining 28 patients and a mean age of 65 years among all 47 patients. Concordant MMR protein loss of the same MMR protein from several tumours from an individual strongly suggests an underlying germline HNPCC-associated mutation, which in 9 cases involved MSH2 and in 8 cases MLH1. Of these patients, 10/17 patients with suspected HNPCC had their first cancer diagnosis after age 50 years, which indicates that the development of metachronous cancers is an important sign of HNPCC and that such patients should not be deferred from genetic analysis because of a high age of onset. The equal distribution of loss of expression in MLH1 and MSH2 in this patient group is in accordance with mutations in these corresponding genes, being found at approximately equal frequencies in HNPCC, although the types of mutations differ somewhat between the genes [8].

Occasional MSS tumours with retained expression for MLH1/MSH2 occurred in 5 of these patients and included a meningioma (case n37), a breast cancer (n45), a sarcoma (n6), a cervical cancer and a vaginal cancer (n46), 3 skin cancers (n47) (Table 2), i.e. tumour types that do not form part of the HNPCC tumour spectrum. Furthermore, 3 MSS colon cancers with retained expression developed in three individuals (n3, n5 and n45) whose remaining tumours generally displayed MSI and concordant loss of expression, of MLH1 or MSH2. This finding suggests that these occa-

sional MSS cancers were indeed sporadic tumours that developed in the HNPCC individuals. Since individuals in the Western world run a 3–5% life-time risk of colorectal cancer, sporadic tumours will also appear in families with hereditary tumours. Although likely to be a rare finding, these cases suggest that when HNPCC is strongly suspected and the genetic analysis does not show MSI and/or immunohistochemical loss, investigation of an additional tumour from the family may be worth considering.

In summary, this study demonstrates a high frequency of MSI and concordant immunohistochemical loss of expression in patients with multiple primary tumours including at least two primary colorectal cancers. Multiple MSI tumours with a concordant loss of MMR protein expression in the tumour tissue, a phenotype strongly suggesting HNPCC, developed in 17/45 (38%) evaluable patients, 10/17 of whom developed the first tumour after age 50 years. Since the Amsterdam II criteria for the classificiation of HNPCC might exclude patients with multiple primary tumours but a weak family history and a higher age at onset, our findings suggest that the development of multiple HNPCC-associated tumours, synchronously or metachronously, in an individual is a strong indicator of HNPCC, irrespective of the age at onset.

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