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ORIGINAL ARTICLE

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Immunohistochemical pattern of hMSH2/hMLH1 in familial and sporadic colorectal, gastric, endometrial and ovarian carcinomas with instability in microsatellite sequences

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Abstract Alterations of DNA mismatch repair (MMR) genes are involved in carcinogenesis of sporadic and inherited human cancers characterised by instability of DNA microsatellite sequences (MSI). MSI tumours are usually identified using molecular analysis. In the present investigation, hMLH1 and hMSH2 immunohistochemistry was tested in order to evaluate the utility of this method in predicting MMR deficiency. Colorectal (72), gastric (68), endometrial (44) and ovarian (17) carcinomas were independently evaluated for familial history, histological type of tumour, MSI status and immunohistochemical results. Loss of expression of either hMLH1 or hMSH2 was observed in 51 of 55 (92.8%) MSI tumours, while 145 of 146 microsatellite stable (MSS) tumours expressed both the hMLH1 and hMSH2 gene products. Independently of tumour site, an overall agreement between immunohistochemical and molecular results was observed in 15 hereditary non-polyposis colorectal cancer-related tumours. Among sporadic tumours, only 2 of 60 colorectal and 2 of 66 gastric carcinomas, displaying MSI, expressed both hMLH1 and hMSH2 gene products. All 39 endometrial and 16 ovarian tumours presented a concordant molecular and immunohistochemical profile. These data show that immunohistochemistry is an accurate and rapid method to predict the presence of defective DNA MMR genes and to identify both sporadic and familial MSI tumours.

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Introduction

Defects in the DNA mismatch repair (MMR) system are involved in carcinogenesis and tumour progression of sporadic and inherited human cancers [10, 26]. MMR deficiency leads to the accumulation of base–base mismatches and short insertion/deletion mispairs, generated as a consequence of DNA replication errors and homologous recombinations. Most cells deficient in MMR often display a high level of genomic instability (MSI-H), characterised by changes in repeat numbers of simple repetitive sequences (microsatellite instability; MSI). In humans, MMR is mediated by at least five genes, including hMLH1, hMSH2, hMSH3, hMSH6 and hPMS2 [23]. Inherited mutations of hMLH1 and hMSH2 genes have been demonstrated as the cause of 70–100% of hereditary non-polyposis colorectal cancers (HNPCC), showing MSI-H [1, 3, 12, 28, 32]. On the contrary, germline hMSH3, hMSH6 and hPMS2 mutations have been rarely identified in HNPCC patients [2, 38].

MMR genes are also involved in the development of a subset of sporadic colorectal, gastric and endometrial tumours. In fact, MSI-H has been observed in 13–44% of gastric [17], 10–15% of colorectal [20, 31] and 17–23% of endometrial [4, 39] sporadic carcinomas. The MSI phenotype, in these cases, is consistent with a somatic MMR defect. Recent studies pointed out that about 90% of sporadic MSI cancers have hMLH1 transcriptional silencing, while a minority of cases show inactivation of hMSH2 or hMLH1 due to somatic mutations [18, 45]. The evidence that epigenetic mechanisms cause inactivation of MMR genes [8] and, in addition, the identification of a high percentage of missense variants of uncertain pathogenetic

significance [16, 38, 54], outline the importance of functional assays in the MMR defect identification.

Immunoistochemical analysis using specific antibodies directed against MMR proteins has proven to be a useful approach to investigate MMR defects, predicting the presence of a defective DNA MMR component both in sporadic and familial MSI tumours [49]. In order to evaluate the utility, sensitivity and specificity of immunoistochemical methods in predicting MMR deficiency, we analysed a large series of familial and sporadic colorectal, gastric, endometrial and ovarian carcinomas comparing MSI status and protein expression pattern.

Materials and methods

Patients and tumour samples

A total of 201 non-consecutive tumours (72 colorectal, 68 gastric, 44 endometrial and 17 ovarian carcinomas; Table 1) from 198 patients were tested for microsatellite (MS) status at mononucleotide loci and for immunohistochemical expression of hMLH1 and hMSH2 gene products. Each case was evaluated independently for familial history, MS status, immunohistochemical reactions and histological type of the neoplasm.

Family histories were carried out during a psychologically assisted genetic counselling with at least a three-generation pedigree reconstruction. Verification of the reported malignancies has been performed using cancer registry, clinical reports, pathological reports, doctor notes and death certificates, as previously described [15]. Pedigree classification was performed as follows: HNPCC type I families were defined according to the Amsterdam criteria [51] and HNPCC type II families were defined according to the recently revised Amsterdam criteria [52]. Endometrial, gastric and ovarian cancers were classified as familial when at least two cases of the same malignant neoplasm were observed in first degree relatives. All remaining cases not fulfilling these criteria were classified as sporadic.

In summary, 1 ovarian, 2 endometrial and 12 colorectal carcinomas arose in 7 HNPCC families. Two gastric carcinomas occurred in the same family, and three endometrial cancers belonged to three families with site-specific endometrial cancers. Tissue samples were fixed in 10% formalin and embedded in paraffin. Sections (5-µm thick) were stained with haematoxylin and eosin (H&E) and periodic acid-Shiff (PAS)-alcian blue stains. Colorectal adenocarcinomas were histologically classified according to the criteria used in a previous study [15], endometrial and ovarian cancers were classified according to the World Health Organization (WHO) criteria [41, 42] and gastric tumours were classified according to Lauren's criteria [27], modified by Solcia et al. [46].

Immunohistochemistry

Immunoperoxidase studies were performed on formalin-fixed paraffin sections that were dewaxed and rehydrated using Bio-clear (Bio-Optica, Milan, Italy) and alcohol. Endogenous peroxidase was blocked by dipping sections in 3% aqueous H_2O_2 for 10 min, and antigen retrieval was performed with a 10-min microwave treatment in 10 mM citrate buffer, pH 6.00. The immunostaining was performed with the avidin–biotin–peroxidase complex technique [19], using diaminobenzidine as a chromogen. Sections were incubated overnight at 4°C with mouse monoclonal antibodies against full-length hMLH1 protein (G168–15, PharMingen, San Diego, Calif.) and the carboxy-terminal fragment of hMSH2 protein (FE11, Oncogene Research Products, Cambridge, Mass.) at 1:100 dilutions. Sections were lightly counterstained with haematoxylin. The normal staining pattern for both hMLH1 and hMSH2 was nuclear, and a case was considered positive only in

Table 1 Summary of the clinico-pathological data of patients. *HNPCC* hereditary non-polyposis colorectal cancers carcinomas; *SCC* sporadic colorectal carcinomas; *FGC* familial gastric carcinomas; *SGC* sporadic gastric carcinomas; *HNPCCEC* HNPCC endometrial carcinomas; *FEC* familial endometrial carcinomas; *SEC* sporadic endometrial carcinomas; *SOC* sporadic ovarian carcinomas; *RC* cecum through splenic flexure; *A* antrum; *CF* corpus and fundus; *C* cardias; *AC* adenocarcinoma; *MUC* mucinous adenocarcinoma; *PDC/UND* poorly differentiated/undifferentiated carcinoma; *INT* intestinal type carcinoma; *DIF* diffuse type carcinoma; *IND* indeterminate or mixed carcinoma; *END* endometrioid carcinoma; *SER* serous adenocarcinoma or cystoadenocarcinoma; *MM* malignant mixed mullerian tumour

	HNPCC (*n*=12)	SCC (*n*=60)	FGC (*n*=2)	SGC (*n*=66)	HNPCCEC (*n*=2)	FEC (*n*=3)	SEC (*n*=39)	HNPCCOC (*n*=1)	SOC (*n*=16)
Mean age (years)±SD	55±14	66±13	36.5	64±10	41	60	69±11	53	56±13
Age range (years)	42–90	35–97	33–40	33–84	35–47	58–62	39–88		28–78
Gender ratio, male/female (%male)	6/6 (50)	29/31 (48)	1/1 (50)	41/25 (62)					
Location of carcinomas, n (%)	R 5 (42)	R 25 (42)	A 2 (100) CF 17 (37) C 6 (9)	A 40 (61)					
Histological type of carcinomas (%)	AC (66) MUC (17) PDC/UND (17)	AC (67) MUC (33)	DIF (50) IND (50)	INT (73) DIF (20) IND (7)	END (100)	END (67) MM (33)	END (72) MUC (2.5) SER (15) PDC/UND (8) MM (2.5)	END (100)	END (12.5) MUC (25) SER (50) PDC/UND (12.5)

the presence of nuclear staining of neoplastic cells. A case was considered negative for expression of hMLH1 or hMSH2 only when there was a complete absence of nuclear staining of neoplastic cells in the presence of an unquestionable internal positive control represented by normal epithelial cells, stromal cells, muscle cells or lymphocytes.

DNA extraction and MSI testing

In 98 cases (33 endometrial, 51 colonic and 14 ovarian carcinomas), DNA was extracted from fresh tumour tissue using QIAamp tissue Kits (Qiagen, Hilden, Germany), and the corresponding normal DNA was obtained from peripheral blood samples using QIAamp blood kits (Qiagen, Hilden, Germany). In 103 cases (11 endometrial, 21 colonic, 68 gastric and 3 ovarian carcinomas), tumour and normal tissue DNAs were obtained from archival paraffin-embedded specimens after microdissection. For these cases, four sequential 5-µm sections were cut from the paraffin-embedded tumour blocks, mounted onto poly-L-lysine coated slides and dewaxed in xylene and ethanol washes. One section was then stained with H&E using standard techniques to confirm the histological identification of the tumour components. Using this slide as a guide, all tumour areas from each block were carefully scraped with a fine scalpel blade from the unstained adjacent sections.

MSI status of each neoplasm was determined through analysis of three mononucleotide repeat markers, including BAT-26, BAT-25 and BAT40. Primers were prepared with the forward primer end labelled with 6-FAM phosphoramidites and were also purified using standard high-performance liquid chromatography (HPLC). Genomic DNA was amplified in a reaction mixture (15 µl) containing 1.5 mM magnesium chloride, 50 mM potassium chloride, 20 mM tris-HCl (pH 8.4), 200 µM of each deoxynucleotide triphosphate, 1 µM of each primer and 2 U *Taq* polymerase (Perkin-Elmer/Cetus, Milan, Italy).

PCR was carried out after a 3-min initial denaturation at 95°C, with 30 cycles of 30 s each of denaturation at 95°C, 30 s of annealing at 55°C and 30 s of elongation at 72°C in a Perkin-Elmer Gene Amp Thermal 2400 (Perkin-Elmer/Cetus, Milan, Italy). A portion of the PCR product was aliquotted and combined with formamide and a GeneScan 2500 internal size marker to permit precise sizing of alleles. Samples were denatured at 94°C and loaded on an Applied Biosystems 310 automated DNA sequencer (Applied Biosystems, Milan, Italy). Fragment sizing analysis was performed using GeneScan 672 (version 1.2) software (Applied Biosystems).

The MSI status of every neoplasm was established according to criteria reported by Zhou et al. [56]. As suggested by these authors, DNA obtained from solid tumours of various organs can be classified as MSI when aberrant alleles with $(A)_{21}$ or less are seen at the BAT-26 locus. The mononucleotide markers BAT-25 and BAT-40 were used to confirm MSI status assayed with BAT-26 marker and to avoid misclassification due to rare BAT-26 normal alleles with $(A)_{25}$ or $(A)_{24}$ tracts. Cases were considered MSI when BAT-25 or BAT-40 were unstable.

Colorectal (50) [15], endometrial (38) [50], gastric (51) and ovarian (16) carcinomas (Furlan, Chiaravalli, Capella, 2000, unpublished data) included in this work were also investigated using the dinucleotide microsatellite markers reported in the previous MSI studies [15, 50]. In these previous analyses, a tumour was considered MSI when instability was observed in at least 20% of the tested loci.

Statistical analysis

The sensitivity, specificity, predictive values for a positive and a negative and overall accuracy of hMLH1 or hMSH2 immunostaining in identifying MSI tumours were calculated.

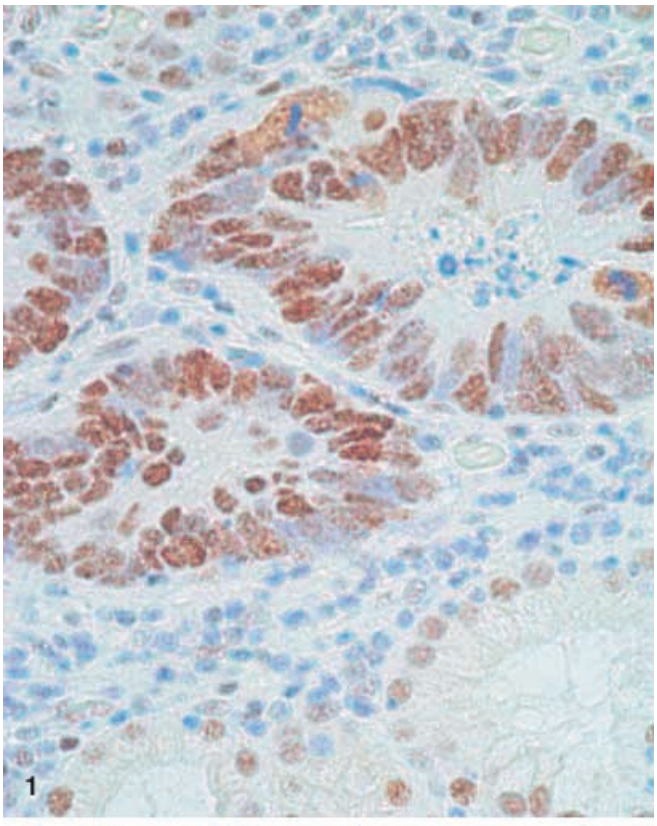
Results

MSI was observed in 55 (27%) of 201 tumours examined. There were 22 colonic, 16 gastric, 13 endometrial and 4 ovarian carcinomas. Of 55 MSI tumour DNAs, 52 were BAT-26 unstable, exhibiting aberrant alleles with losses ranging from 5 to 15 nucleotides. The remaining three tumour DNAs (from one gastric and two endometrial cancers) exhibited losses ranging from one to four nucleotides. Since these cases showed instability of the BAT-25 or BAT-40 markers, they were considered as MSI [9].

At the Bat-26 locus, all 146 MSS tumour DNAs exhibited the normal allele $(A)_{26}$ and no microsatellite alterations at BAT-25 or BAT-40 loci. Of the 55 MSI cases previously investigated [15, 50] and included in this work, 51 showed instability in more than 20% of tested loci and, therefore, were classified as MSI. The remaining four cases (two colorectal and two gastric cancers) showing MSI in less than 20% of tested loci were previously considered MSS.

The immunohistochemical evaluation of tumour samples needed a careful examination because of the presence of positive stromal fibroblast and lymphoid elements within the tumour and the variable intensity of nuclear stain of tumour cells. In some cases, usually histologically processed before 1985, we observed areas with different intensity of immunohistochemical reaction of both tumour and stromal elements, probably due to prolonged formalin fixation. Therefore, a case was considered negative for the expression of one of the two gene products only when all neoplastic cells were negative in presence of normal intermingled positive cells. In cancers retaining nuclear expression of both hMLH1 and hMSH2 gene products, the nuclear staining of tumour cells was generally more intense than that of infiltrating lymphocytes, stromal cells and normal epithelial cells adjacent to the tumour (Fig. 1). In addition, we also observed an intense immunohistochemical reaction for both gene products in the germinal centre cells of lymphoid follicles or in epithelial cells located in the proliferative zones of normal gastric and intestinal glands. Both of these findings suggest that the nuclear accumulation of hMLH1 and hMSH2 gene products is increased in cells more rapidly proliferating (neoplastic versus normal intermingled cells and cells of proliferative zones of normal tissues versus quiescent cells). In all cases showing loss of nuclear expression of hMLH1 or hMSH2 gene products in tumour cells, there were positive non-neoplastic elements admixed with neoplastic cells and/or adjacent to the tumour (Fig. 2).

The overall immunohistochemical and molecular results are summarised in Table 2. Considering all 201 tumours independently of tumour site and familial history, 52 (25.9%) cases showed no expression of one of two gene products studied. All of the other 149 (74.1%) cases showed nuclear staining of tumour cells for both proteins. Loss of expression for either hMLH1 or



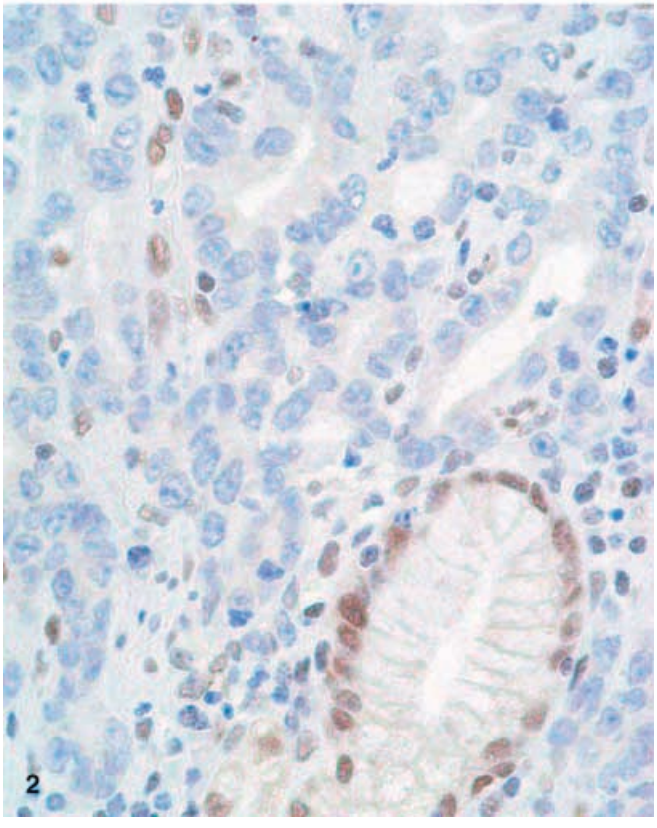


Fig. 1 Microsatellite-stable gastric carcinoma with intense nuclear staining of tumour cells for hMSH2. The immunoreactivity of tumour cells is more intense than that of adjacent residual normal glandular structures and admixed lymphocytes (×400)

Table 2 Correlation between immunohistochemical results and molecular profile of 201 carcinomas examined. *MSI* microsatellite instable; *MSS* microsatellite stable

	Number of cases	Expression of hMLH1 and hMSH2 present	Expression of hMLH1 or hMSH2 absent
MSI	55	4 (7.2%)	51(92.8%)
MSS	146	145 (99.3%)	1 (0.7%)
Total	201	149 (74.1)	52 (25.9)

hMSH2 was observed in 51 of 55 (92.8%) MSI tumours, while 4 of 55 (7.2%) MSI tumours (two gastric and two colorectal carcinomas) showed mononucleotide instability but intense immunoreactivity for both proteins. These four cases were tested for hMSH6 immunoreactivity using the MSH6/GTBP monoclonal antibody (Clone 44, Transdaction Lab., San Diego, Calif.). Three cases were hMSH6 positive, while the fourth case was not valuable (Chiaravalli A.M., unpublished results).

Of 146 MSS tumours, 145 (99.3%) showed immunoreactivity for both hMLH1 and hMSH2 gene products, while one case was negative for hMSH2. This MSS tumour, with negative immunohistochemical reaction for hMSH2, was a small intramucosal early gastric cancer composed of signet ring cells interspersed with numerous residual normal glandular cells. In summary, the absence of nuclear staining for the hMLH1 or hMSH2 gene product had a sensitivity of 92.7% for MSI status, a specificity of 99.3%, a predictive value of 98.1% for a positive, a predictive value of 97.3% for a negative and an overall accuracy of 97.5%.

The immunohistochemical and molecular results correlated with family history and tumour site are summarised in Table 3. Considering carcinomas in HNPCC-patients, 11 of 15 (73%) tumours (one ovarian, two endometrial and eight colorectal cancers) showed both MSI and absence of immunohistochemical expression of either hMLH1 or hMSH2 gene products. In particular, hMLH1 was not expressed in four of eight colorectal carcinomas, one of two endometrial carcinomas and in the only one ovarian cancer, while hMSH2 was absent in the remaining one endometrial and four colorectal carcinomas. Among these HNPCC patients (four families), hMLH1 germline mutations were confirmed using single-strand conformation polymorphism (SSCP) analysis in two families with tumours showing absence for hMLH1 expression (Dr. G Guanti, Medical Genetic Dept., University of Bari, personal communications). The other two families are ongoing. The remaining four HNPCC tumours were all MSS colorectal carcinomas and displayed an intense nuclear staining for both hMLH1 and hMSH2. With regard to HNPCC associated

Fig. 2 Microsatellite gastric carcinoma negative for hMLH1. Immunoreactivity is present in glandular and stromal cells admixed with tumour cells (×400)

Table 3 Summary of immunohistochemical and molecular results correlated with familial setting and site of the tumours. *MS* microsatellite; *MSI* microsatellite instable; *MSS* microsatellite stable; HNPCC hereditary non-polyposis colorectal cancers

	MS status	Expression of hMLH1 and hMSH2 present	Expression of hMLH1 or hMSH2 absent	Expression of hMLH1 absent	Expression of hMSH2 absent
Colorectal carcinomas					
12 HNPCC	8 MSI	0	8 (100%)	4 (50%)	4 (50%)
	4 MSS	4 (100%)	0		
60 sporadic	14 MSI	2 (14.3%)	12 (85.7%)	12 (100%)	0
	46 MSS	46 (100%)	0		
Gastric carcinomas					
2 familial	2 MSS	2 (100%)	0		
66 sporadic	16 MSI	2 (12.5%)	14 (87.5%)	13 (92.9%)	1 (7.1%)
	50 MSS	49 (98%)	1 (2%)	0	1
Endometrial carcinomas					
2 HNPCC	2 MSI	0	2 (100%)	1(50%)	1(50%)
3 familial	3 MSS	3 (100%)	0		
39 sporadic	11 MSI	0	11 (100%)	9 (81.8%)	2 (18.2%)
	28 MSS	28 (100%)			
Ovarian carcinomas					
1 HNPCC	1 MSI	0	1 (100%)	1 (100%)	0
16 sporadic	3 MSI	0	3 (100%)	3 (100%)	0
	13 MSS	13 (100%)	0		

Table 4 Correlation between microsatellite (MS) status and histological types of tumours. *AC* Adenocarcinoma; *MUC* mucinous adenocarcinoma; *PDC/UND* poorly differentiated/undifferentiated carcinoma; *INT* intestinal type carcinoma; *DIF* diffuse type carcinoma; *IND* indeterminate or mixed carcinoma; *END* endometrioid adenocarcinoma; *SER* serous adenocarcinoma or cystoadenocarcinoma; *MM* malignant mixed mullerian tumour; *MSI* microsatellite instable; *MSS* microsatellite stable

	MS status	Number of cases	Histotype				
Colorectal carcinomas			AC	MUC	PDC/UND		
HNPCC	MSI	8	4 (50%)	2 (25%)	2 (25%)		
	MSS	4	4 (100%)				
Sporadic	MSI	14	6 (43%)	8 (57%)			
	MSS	46	34 (74%)	12 (26%)			
Gastric carcinomas			INT	DIF	IND		
Familial	MSS	2		1	1		
Sporadic	MSI	16	14 (88%)	1 (6%)	1 (6%)		
	MSS	50	34 (68%)	12 (24%)	4 (8%)		
Endometrial carcinomas			END	MUC	SER	PDC/UND	MM
HNPCC	MSI	2	2 (100%)				
Familial	MSS	3	2 (67%)				1 (33%)
Sporadic	MSI	11	9 (82%)	1 (9%)		1 (9%)	
	MSS	28	19 (68%)		6 (21%)	2 (7%)	1 (4%)
Ovarian carcinomas			END	MUC	SER	PDC/UND	MM
HNPCC	MSI	1	1	1			
Sporadic	MSI	3	1 (33%)	2 (67%)			
	MSS	13	1 (8%)	2 (15%)	8 (62%)	2 (15%)	

tumours, hMLH1 and hMSH2 immunohistochemical technique showed 100% sensitivity and 100% specificity in detecting alterations in the MMR system.

Regarding histological type of the 11 MSI tumours, the colorectal carcinomas were represented by four well- or moderately differentiated adenocarcinomas, two mucinous and two poorly differentiated/undifferentiated adenocarcinomas, the endometrial tumours were represented by two endometrioid adenocarcinomas and the ovarian tumours were represented by one endometrioid adenocarcinoma. The four MSS tumours were all colorectal well- or moderately differentiated adenocarcinomas (Table 4).

The immunohistochemical analysis of the 14 MSI colorectal carcinomas representing 23.3% of all 60 sporadic colorectal cases examined revealed the absence of nu-

Table 5 Correlation between absence of expression of hMLH1/hMSH2 and histological types. *AC* Adenocarcinoma; *MUC* mucinous adenocarcinoma; *PDC/UND* poorly differentiated /undifferentiated carcinoma; *INT* intestinal type carcinoma; *DIF* diffuse type carcinoma; *IND* indeterminate or mixed carcinoma; *END* endometrioid adenocarcinoma; *SER* serous adenocarcinoma or cystoadenocarcinoma; *MM* malignant mixed mullerian tumour

	Negative for	Number of cases	Histotype				
Colorectal carcinomas			AC	MUC	PDC/UND		
HNPCC	hMLH1	4	2 (50%)	1 (25%)	1 (25%)		
	hMSH2	4	2 (50%)	1 (25%)	1 (25%)		
Sporadic	hMLH1	11	4 (37%)	7 (63%)			
	hMSH2	1		1			
Gastric carcinomas			INT	DIF	IND		
Sporadic	hMLH1	13	11 (84%)	1 (8%)	1 (8%)		
	hMSH2	2	1 (50%)	1 (50%)			
Endometrial carcinomas			END	MUC	SER	PDC/UND	MM
HNPCC	hMLH1	1	1				
	hMSH2	1	1				
Sporadic	hMLH1	9	7(78%)	1(11%)		1(11%)	
	hMSH2	2	2				
Ovarian carcinomas			END	MUC	SER	PDC/UND	MM
HNPCC	hMLH1	1	1				
Sporadic	hMLH1	3	1 (33%)	2 (67%)			

clear staining in 12 cases (11 for hMLH1 and 1 for hMSH2) and expression of both gene products in two cases. The 46 MSS tumours representing 76.7% of all sporadic cases examined showed immunoreactivity for both hMLH1 and hMSH2 gene products. Histologically, MSI sporadic colorectal cancers were represented prevalently by mucinous adenocarcinomas (8 of 14; 57%), while the majority of MSS tumours were well- or moderately differentiated adenocarcinomas (34 of 46; 74%; Table 4). Of the 11 hMLH1 negative cases, four (37%) were well- or moderately differentiated adenocarcinomas, and the remaining seven (63%) cases were mucinous adenocarcinomas. The hMSH2 negative tumour was a mucinous adenocacarcinoma (Table 5).

Among the 16 MSI (24.2% of all sporadic cases examined) gastric carcinomas, 13 carcinomas revealed an absence of nuclear staining for hMLH1 and one for hMSH2. The remaining two MSI cases were immunoreactive with both hMLH1 and hMSH2 antibodies. The 52 MSS (75.8% of all sporadic cases and the two familial cases) gastric cancers were positive for both hMLH1 and hMSH2, except for one case, represented by the early gastric cancer described above. Regarding the histological type, 14 of 16 (88%) MSI tumours were intestinal type carcinomas, while the remaining two cases were represented by a diffuse-type carcinoma and an indeterminate type carcinoma, respectively (Table 4). The absence of nuclear expression of the hMLH1 gene product was observed prevalently in intestinal type carcinomas (11 of 13 cases; 84%), while hMSH2 was not expressed in one diffuse type and in one intestinal type carcinoma (Table 5). Among sporadic MSS cases, the diffuse histotype was observed in 12 of 50 (24%) carcinomas, while the intestinal type was observed in 34 of 50 (68%) carcinomas.

Among endometrial carcinomas, apart from those of the HNPCC patients described above, MSI was observed in 11 cases (28.2% of all sporadic cases). Histologically, MSI endometrial cancers were represented by one mucinous and nine endometrioid adenocarcinomas and one undifferentiated carcinoma (Table 4). Among these MSI tumours, the expression of hMSH2 or hMLH1 gene products was absent in two and nine cases, respectively (Table 5). All of the 31 (71.8% of all sporadic cases and the three familial cases) MSS tumours revealed intense nuclear staining of tumour cells for both hMLH1 and hMSH2 gene products (Table 3). Regarding histological type, the sporadic MSS tumours included 19 endometrioid, 6 serous adenocarcinomas, 2 undifferentiated carcinomas and one malignant mixed mullerian tumour.

Of 16 sporadic ovarian cancers, three (18.7%) showed MSI and no nuclear staining for hMLH1 antibody, while expression of both proteins was observed in the other 13 MSS tumours. The MSI carcinomas included two mucinous cystoadenocarcinomas and one endometrioid adenocarcinoma, while the prevalent histotype of MSS tumours was the serous cystoadenocarcinoma (8 of 13 cases; 62%).

Discussion

The assessment of MMR defects has become an important tool in tumour molecular pathology and clinical practice. MSI and MSS phenotypes appear to characterise two different pathways of carcinogenesis. Patients who present with colorectal [33, 48] or gastric [37] MSI carcinomas have a better prognosis than those with MSS tumours. The status of MMR may be important in pre-

dicting tumour response to clinical therapy [40]. Finally, it is now widely accepted that the assessment of MMR defects should be added as a distinct criterion to define the diagnosis of HNPCC.

Germline mutations in hMSH2 and hMLH1 account for about 90% of all reported MMR gene mutations; hPMS2 and hMSH6 account for the remainder [30]. With regard to sporadic colorectal, endometrial and gastric tumours, 90% of MSI-H cases have transcriptional silencing of hMLH1, and the remainder are consistent with inactivation of hMSH2 or hMLH1 by somatic mutations [18, 45].

The identification of pathogenetic alterations in hMLH1 or hMSH2 often becomes complicated by the absence of hot-spot mutations for these genes, missense variants of uncertain pathogenic significance and by epigenetic mechanisms, such as methylation that cause gene silencing. Recently, in a series of HNPCC patients, it was demonstrated that the absence of hMLH1 and hMSH2 immunohistochemical expression may identify tumours with MMR deficiency [36, 49]. Molecular analysis is normally used to identify sporadic and familial MSI tumours, but it is difficult, expensive and often conditioned by the availability of tumour samples, which have been correctly collected and conserved. In addition, the molecular analysis for MSI does not allow the identification of the gene involved, which requires further molecular examinations.

To verify reliability of interpretation, sensitivity and specificity of hMLH1 or hMSH2 immunostaining in predicting MSI, we studied a large series of familial and sporadic colonic, gastric, endometrial and ovarian carcinomas using both immunohistochemical and molecular analyses. Our data confirm a strong correlation of MSI with loss of expression of either hMSH2 or hMLH1 as reported in previous studies [5, 36]. The immunohistochemical analysis showed high sensitivity (92.8%), specificity (99.3%), predictive value for a positive (98.1%) and predictive value for a negative (97.3%) in demonstrating deficiency of the MMR system. The higher correlation between immunohistochemical and molecular results observed in our study relative to others [6, 47] might be due to the use of different hMLH1 monoclonal antibodies. In our experience, the clone G168-15 has been shown to be more sensitive and reproducible than the clone G168-728 used by other authors (Chiaravalli A. M., unpublished observations).

With regard to specificity in all but one cases analysed, the lack of expression of one of the two gene products corresponded with the presence of MSI. The only case that resulted MSS but hMLH1 negative was a small early gastric cancer in which it was difficult to outline tumour areas for DNA extraction because neoplastic cells were admixed with normal mucosal elements and the percentage of tumour resulted lower than 50%. If we would shut out this case from the study, because of a technical problem, we could obtain a 100% specificity. It is noteworthy in cases like this that, in identifying MSI, the immunohistochemical technique is a more efficient method than a molecular approach, which needs a laser microdissection in order to avoid the presence of a high percentage of non-tumour cells.

With regard to sensitivity, 51 of 55 (92.8%) MSI tumours lacked one of the gene products. In tumours from HNPCC patients, independent of tumour site, MSI was directly associated with the lack of expression of one of the two proteins tested. These data are in agreement with a previous study suggesting, in these tumours, the presence of underlying germline mutations of either hMLH1 or hMSH2 [36]. On the contrary, among sporadic cancers retaining nuclear expression of hMLH1 and hMSH2 gene products, four cases were MSI. These data are consistent with previous findings in which MSI was observed in cases without detectable mutations in hMLH1 and hMSH2 and with normal immunohistochemical staining for these proteins [49], suggesting that other genes affecting MMR function may play a pathogenic role. In addition, Yamamoto et al. [55] recently demonstrated hMSH3 or hMSH6 mutations in gastric MSI tumours without alterations of hMLH1 or hMSH2 genes. In particular, these above-mentioned four cases were the two colorectal and two gastric cancers with only mononucleotide instability (see Results). In these cases, other MMR genes could be involved. In fact, in a recent study, germinal hMSH6 mutations were found in families with less penetrant aggregations of colon cancer that did not meet Amsterdam criteria and displayed an attenuated form of MSI involving only selected mononucleotide repeats [14, 53].

In contrast to HNPCC tumours, the majority of our sporadic tumours are characterised by the absence of hMLH1 gene product expression (91.7% colorectal, 92.9% gastric, 81.8% endometrial and 100% ovarian carcinomas), suggesting that alterations of hMLH1 protein are more frequently involved in MSI of sporadic tumours. In fact, Simpkins et al. [45] and Esteller et al. [11] proposed hMLH1 promoter methylation and gene silencing as a primary cause of MSI in sporadic endometrial cancer, providing that the hMSH2 gene may contribute substantially to inherited forms. Hypermethylation of the hMLH1 gene promoter and associated reduced expression of hMLH1 protein was also demonstrated in gastric [13, 29] and colorectal [18] sporadic cancers.

With regard to HNPCC tumours, the immunohistochemical screening is an efficient and sensitive method, showing the absence of MSH2/MLH1 protein in all MSI cancers (1 ovarian, 2 endometrial and 8 colorectal cancers), and defects of the two MMR proteins were equally involved in our MSI-positive families. Interestingly, in each HNPCC family, the pattern of MSI and of MSH2 and MLH1 gene expression was homogeneous in carcinomas of all sites. On this ground, this approach represents a suitable method to identify HNPCC families through characterisation of tumours from different sites involved in the HNPCC spectrum. In addition, the combined immunohistochemical and molecular assays may be helpful to avoid phenocopies and genetic

heterogeneity, to address the screening of germline mutations and to identify familiar cancers carrying germline mutations of unknown genes, such as modifier genes [8].

Correlating histological types with MS status and immunohistochemical expression of hMSH2 and hMLH1, we can note that, with regard to sporadic tumours, MSI and the absence of expression of hMLH1 or hMSH2 proteins were observed more frequently in mucinous adenocarcinomas of the colorectum and of the ovary, intestinal type carcinomas of the stomach and endometrioid adenocarcinomas of the endometrium and of the ovary. In addition, poorly differentiated/undifferentiated carcinomas of both the large bowel and the endometrium, but not undifferentiated ovarian carcinomas, were found to be MSI tumours.

Among HNPCC colorectal cancers, the prevalent histotype of both MSI and MSS tumours was adenocarcinoma. On the contrary, among sporadic cases, MSI tumours were prevalently represented by mucinous adenocarcinomas (57%), while MSS tumours consisted prevalently of adenocarcinomas (74%). These data, in agreement with previous studies [22, 25, 35], seem to confirm that alterations of the MMR system are more frequently involved in the pathogenesis of sporadic colorectal mucinous adenocarcinomas [15], intestinal carcinomas of the stomach [34, 43] and endometrioid adenocarcinomas of the endometrium [50]. Controversial data about the frequency and the histological type of MSI ovarian cancers are reported in the literature [21, 44]. In our study, MSI ovarian carcinomas were only of endometrioid or mucinous type.

Considering gene expression, we can note that, while hMSH2 protein is about equally absent in the different types of colorectal and gastric carcinomas, the absence of immunostaining for hMLH1 is observed more frequently in mucinous colorectal adenocarcinomas and intestinal-type gastric carcinomas than in colorectal adenocarcinomas and diffuse-type carcinomas of the stomach. Probably, in sporadic mucinous colorectal adenocarcinomas and in gastric intestinal-type carcinomas, MSI is more frequently related to methylation of the hMLH1 gene promoter than to mutations of the genes [7, 13, 18, 24].

In conclusion, an immunohistochemical technique, predicting with accuracy the presence of a defective DNA MMR components, is a good, rapid and cheap strategy to screen for MSI tumours. In addition, it has an immediate application in the clinical practice of identifying MSI sporadic tumours and of detecting possible cases of HNPCC cancers.

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