

Immunohistochemistry for MSH2 and MHL1: a method for identifying mismatch repair deficient colorectal cancer

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Abstract

Colorectal cancers with DNA mismatch repair (MMR) gene mutations characteristically display a high rate of replication errors in simple repetitive sequences detectable as microsatellite instability (MSI). Most are the result of somatic MMR dysfunction; however, a subset are caused by germline mutations. The availability of commercial antibodies for MSH1 and MLH2 offers an alternative strategy to molecular methods for identifying MMR deficient cancers. To evaluate immunohistochemistry, MLH1 and MSH2 expression was studied using monoclonal antibodies in formalin fixed, paraffin wax embedded cancers. The immunohistochemical staining patterns of 23 cancers displaying MSI, including four cases with germline mutations, were compared with 23 microsatellite stable (MSS) cancers. All MSS cancers exhibited staining with both antibodies. Twenty two of the MSI cases showed absent MMR expression with either anti-MSH1 or anti-MLH2. The high sensitivity and predictive value of immunohistochemistry in detecting MMR deficiency offers a method of discriminating between MSI and MSS cancers caused by MSH1 and MLH2 dysfunction. The application and suitability of immunohistochemistry for the detection of MSI and as a strategy for prioritising the mutational analysis of MMR genes in routine clinical practice is discussed.

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Keywords: colorectal cancer; mismatch repair; MSH2; MLH1; hereditary non-polyposis colorectal cancer

The human DNA mismatch repair (MMR) system, responsible for correcting DNA mismatches arising during replication, comprises at least six genes, with the principal ones being MSH1 and MLH2. Inherited deficiency of MMR function underlies the hereditary non-polyposis colorectal cancer (HNPCC) syndrome.¹ Approximately 80% of HNPCC can be ascribed to germline mutations in either MHL1 or MSH2.¹

The study of MMR deficient cancers from individuals affected with HNPCC has led to the recognition that MMR inactivation provides an alternative pathway for colorectal carcinogenesis. MMR deficient cancers are not typified by chromosomal instability and exhibit low rates of allele loss. Morphologically they are often polypoidal, mucinous, and

undifferentiated and display extensive necrosis.² DNA mismatch repair deficiency is also a feature of approximately 15% of sporadic colorectal cancers; in general, as a result of hypermethylation of the promoter sequence, but sometimes as a result of somatic mutations.^{3,4}

The biological behaviour of colorectal cancers with MMR deficiency appears to be distinct from those with intact MMR.² Paradoxically, despite a high risk of metachronous disease in patients harbouring germline mutations, the prognosis appears to be better than for patients with MMR competent tumours.⁵ This also appears to be a feature of sporadic colorectal cancers that are MMR deficient. In addition, there is experimental evidence indicating that tumours deficient for MMR respond differently to chemotherapy.⁶ MMR deficient cells are highly tolerant to methylating drugs such as streptozocin and temozolomide and, to a lesser extent, to cis-platin and doxorubicin.⁶ Although there is little direct evidence from trials in humans, it seems plausible that the MMR status of tumours may become an important determinant in the choice of chemotherapeutic intervention.

Distinguishing colorectal cancers that exhibit MMR deficiency provides a method of refining the identification of individuals harbouring germline mutations. Furthermore, it provides a means of identifying patients with colorectal cancer in whom the behaviour of the disease will be different. The increased rate of spontaneous point mutations and high frequency of deletion/insertion mutations in short repetitive DNA that characterises these MMR deficient cancers offers a method of identifying cases through the detection of microsatellite instability (MSI). However, establishing MSI is not simple, and requires access to molecular diagnostic facilities. The recent availability of commercial antibodies for MSH1 and MLH2 potentially offers a more straightforward approach to identifying MMR deficient colorectal cancers. To assess the usefulness of this approach we have undertaken a study of 46 colorectal cancers.

Materials and methods

PATIENTS AND TUMOUR SAMPLES

For our study, we made use of an ongoing survey of the frequency of MMR germline mutations in early onset colorectal cancer (patients

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Table 1 MSI and MMR gene mutation status and MLH1 and MSH2 protein expression in the 46 colorectal cancers studied

Patient	MSI status	Germline mutation	MLH1 expression	MSH2 expression
2142	+	+*	-	+
4046	+	-	-	+
2140	+	-	+	-
4020	+	-	+	-
4002	+	-	-	+
2055	+	-	-	+
2112	+	-	-	+
2217	+	-	+	+
4088	+	+†	-	+
2181	+	-	-	+
2047	+	-	-	+
2088	+	-	-	+
2065	+	-	-	+
2185	+	-	+	-
2204	+	+‡	-	+
2137	+	-	+	-
140	+	-	-	+
2183	+	-	-	+
331	+	+¶	-	+
4094	+	-	+	-
82	+	-	-	+
70	+	-	-	+
4171	+	-	-	+
2020	-	-	+	+
2045	-	-	+	+
2090	-	-	+	+
2196	-	-	+	+
2127	-	-	+	+
2109	-	-	+	+
2154	-	-	+	+
409	-	-	+	+
386	-	-	+	+
382	-	-	+	+
337	-	-	+	+
88	-	-	+	+
2174	-	-	+	+
2205	-	-	+	+
4053	-	-	+	+
2193	-	-	+	+
2209	-	-	+	+
2091	-	-	+	+
2015	-	-	+	+
2037	-	-	+	+
2085	-	-	+	+
2084	-	-	+	+
2201	-	-	+	+

*MLH1 exon 6, codon 175, 10 bp deletion; †MLH1 exon 19, codon 753, T deletion; ‡MLH1 exon 13, codon (CGA to TGA) 487 stop; ¶MLH1 exon 17, codon 646, AG insertion. MMR, DNA mismatch repair; MSI, microsatellite instability.

less than 55 years old at diagnosis) systematically ascertained through three regional UK cancer registries. Formalin fixed, paraffin wax embedded blocks of colorectal cancers and EDTA venous blood samples were obtained from each patient. Our study was undertaken with approval from the relevant local ethics committee.

MOLECULAR ANALYSES

MSI was assessed using a fluorescent polymerase chain reaction (PCR) based assay at the following loci: D1S508, D2S123, D3S1561, D5S346, D11S29, TGFBIIR, D15S970, DCC, D19S565, BAT25, and BAT26. Microsatellite instability was defined as the presence of altered allele sizes in the PCR amplified product of tumour DNA compared with normal DNA. Tumours were designated as MSI positive if altered bands were seen in two or more microsatellites. The full coding sequences and splice junctions of human MLH1 (hMLH1) and hMSH2 were amplified using the PCR. Both PCR primers were end labelled with γ [³²P] ATP using T4 polynucleotide kinase and the amplified fragments were analysed by conformation sensitive gel

electrophoresis.⁷ PCR products from samples that showed migration shifts were directly sequenced in forward and reverse directions and analysed on ABI377 DNA sequencers. Mutations were numbered according to accepted conventions.

IMMUNOHISTOCHEMISTRY

For each colorectal cancer, eight sections (3–4 μ m thick) were cut and mounted on to glass slides. After dewaxing and rehydration of sections, antigenic site retrieval was accomplished by microwaving each slide for five minutes in 0.01 M citric acid buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation with 2% hydrogen peroxide for 20 minutes and non-specific binding prevented by incubation with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Sections were subsequently incubated with either monoclonal anti-MSH2 or anti-MLH1 antibodies (Oncogene, Cambridge, Massachusetts, USA) for two hours at room temperature. Antibody binding was detected using the Elite Vectastain ABC kit (Vector Laboratories Ltd, Peterborough, UK), which is based on the biotin-avidin system, using the manufacturer's protocol. The reaction was visualised using a VIP substrate kit for peroxidase (Vector Laboratories Ltd). Sections were then dehydrated and mounted. Normal colorectal tissue adjacent to the carcinoma was used as the positive control. Loss of expression was recorded when nuclear staining was observed in normal tissue but not in adjacent malignant cells.

Results

Table 1 shows the MSI and germline MSH1 and MLH2 status of the 46 colorectal cancers studied. Also shown are the immunohistochemical results for the anti-MSH1 and anti-MLH2 antibodies. The scoring of both antibodies was essentially straightforward. Intact nuclear staining of tumour cells with antibodies to both MSH1 and MLH2 was seen in all of the 23 MSI negative colorectal cancers, concordant with the molecular analysis indicating that these tumours had no evidence of MMR deficiency. In contrast, 22 of the 23 cancers displaying MSI showed no nuclear staining for one of the MMR proteins, permitting the underlying gene inactivation to be inferred (table 1).

The four colorectal cancers harbouring germline mutations in MLH1 were correctly identified. Of the MSI positive colorectal cancer cases that did not harbour a germline MMR mutation there was a preponderance of MLH1 deficiency compared with MSH2 (2.8 : 1). No tumour showed loss of staining with both anti-MLH1 and anti-MSH2 antibodies. These observations of MSH1 and MLH2 staining suggest that immunohistochemistry identifies MMR deficiency with 96% sensitivity (95% confidence limits (CL), 90% to 100%). The negative predictive value of 96% (95% CL, 90% to 100%) reflects the fact that one tumour exhibited MSI but showed staining for both antibodies.

Table 2 Summary statistics for our study and published reports

Reference	N	Cancers studied	Antibodies used		% Sensitivity (95% CL)	Specificity (%)	% PPV	% NPV
			MLH1	MSH2				
Thibodeau <i>et al</i> (1996) ⁸	26	5 cancers with germline MLH1 mutations 3 cancers with germline mutations 11 MSI cancers 7 MSS cancers	PharMingen G168-728	Oncogene FE11	72 (55 to 89)	100	100	50 (39 to 78)
Marcus <i>et al</i> (1999) ⁹	72	5 cancers with germline MHL1 mutations 11 cancers germline MHS2 mutations 22 MSI cancers 34 MSS cancers	PharMingen G168-728	Oncogene FE11	97 (93 to 100)	100	100	97 (93 to 100)
Dieumegard <i>et al</i> (2000) ¹⁰	31	5 germline MLH1 mutations carriers 3 germline MSH2 mutations carriers 6 MSI cancers 17 MSS cancers	Oncogene Clone Ab-1	Oncogene Clone FE11	77 (61 to 92)	100	100	85 (72 to 98)
Our present study	46	4 germline MLH1 carriers 19 MSI cancers 23 MSS cancers	Oncogene	Oncogene Clone FE11	96 (90 to 100)	100	100	96 (90 to 100)

CL, confidence limit; MSI, microsatellite instability; MSS, microsatellite stable.

Table 2 shows summary statistics for our study and other recently published studies that have evaluated the use of immunohistochemistry as a method of defining the MMR status of colorectal cancers.

Discussion

Colorectal cancers displaying MMR deficiency are characterised by a distinctive morphological and clinical phenotype. Testing cancers for MMR deficiency provides a method of delineating a subset of sporadic cancers with a different clinical course and possible difference in response to chemotherapy.^{5,6} Furthermore, and more importantly, testing colorectal cancers for this phenotype offers a means of refining the identification of patients harbouring germline mutations. The high risk of cancer conferred by constitutional mutations in the MMR genes (an approximate 70% risk of colorectal cancer and a pronounced increase in the risk of uterine and other adenocarcinomas)¹ necessitates the long term follow up and screening of carriers.

The screening of individuals with colorectal cancer for germline MMR mutations is currently undertaken in cases compatible with a hereditary basis (young onset, right sided, dominant inheritance of colorectal cancer). However, the analysis of constitutional DNA for MSH1 and MLH2 mutations is not straightforward because both genes are relatively large and mutations are scattered throughout each gene; hence, identification of MSI in tumours provides a practical method of identifying those patients in whom mutational analysis is appropriate. The detection of MSI in colorectal cancers classically requires microdissection and access to molecular biological facilities. Thus, it is a relatively complex procedure, expensive, and labour intensive, and is therefore not ideally suited to routine clinical practice. Furthermore, it does not circumvent the issue of screening more than one MMR gene for mutations.

In our study, we have evaluated the usefulness of immunohistochemistry as a method of

identifying colorectal cancers with MMR deficiency as a result of the inactivation of the MSH1 or MLH2 gene. We found the sensitivity of this approach to be over 90%, which supports the findings of other recently published studies (table 2). Therefore, immunohistochemistry appears to be a good surrogate test for the detection of MSI caused by MSH2 and MLH1 dysfunction. One caveat to this is that, because of the interdependency between MMR genes, absent or reduced protein expression may be a consequence of the disruption of an interacting MMR gene. The other is that the demonstration of normal protein expression does not entirely preclude gene disruption because mutations that have pathological consequences through RNA decay or other similar mechanisms will go undetected. Therefore, testing cancers for MSI will still be required in cases where there is a high probability of HNPCC but protein expression is present.

Although immunohistochemistry alone cannot be relied upon to distinguish MSI colorectal cancers, it offers an additional method of prioritising mutation analyses suited to routine clinical laboratory practice. Because a small number of MSI positive colorectal cancers are caused by mutations in MMR genes other than MSH1 and MLH2,¹ the usefulness of immunohistochemistry as an initial screening tool will be extended by the use of antibodies against MSH6, PMS1, and PMS2.

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Correspondence

Small cell melanoma

In their recent paper on small cell malignant melanoma,¹ Blessing and co-workers report a series of 15 melanocytic lesions that, on the basis of their histology, were considered to constitute a new variant of naevoid melanoma—melanoma resembling naevus.

I am concerned about the lack of metastases in the reported series. Only documentation of metastasis constitutes formal proof that the lesions are diagnosed correctly as melanomas; histological resemblance to some features of melanoma by itself can never provide the necessary conclusive evidence. In addition, I cannot agree with the authors that some of the features of these lesions—such as vascular proliferation, lymphocytic infiltrate, and lentiginous junctional component—constitute supportive evidence of malignancy. Furthermore, the authors point out that in some respects the lesions resembled benign naevi; it is unclear why the resemblance to melanoma would be more relevant than the resemblance to naevus. From the illustrations provided in the paper, I am not sure that I would issue a confident diagnosis of melanoma.

One needs to have more cases with follow up to obtain a better picture of the possible malignant potential of these lesions. If no metastases are encountered in an expanded series, then the message of the paper would be very different and similar to the one of Sophie Spitz in her classic paper on what was then termed juvenile melanoma²: the lesions under study resemble melanoma in some respects, but are devoid of malignant potential. Careful correlation of histology with follow up data in a large series is the only way to solve this issue and to know how to interpret such lesions correctly.

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The authors reply

We would like to thank Professor Mooi for taking an interest in our recent article describing small cell melanoma as a variant of classic melanoma.¹ The points that he makes are of course entirely relevant and are, we feel, generally covered in the manuscript; indeed, the title reflects the fact that the diagnosis might be contentious. Therefore, only the main points of his letter will be discussed.

He rightly comments that there is a lack of metastases in our cases. However, apart from one lesion that measured 1.1 mm, all were less than 1.0 mm, with a mean of 0.63 mm, and therefore the clinical behaviour of these cases is not unexpected. It is of course possible that “small cell melanoma” may have an inherently less aggressive behaviour. In addition, although we accept that metastasis is the

gold standard for the diagnosis of malignancy, histopathologists readily accept basal cell carcinoma as a malignant epithelial neoplasm without metastatic potential and, indeed, those who accept the concept of the radial growth phase in melanoma are quite happy to call these potentially non-metastasising lesions melanomas.

Professor Mooi expresses discontent over the features such as lentiginous junctional growth pattern, lymphohistiocytic infiltrate, and vascular proliferation as being supportive of malignancy. We agree that no feature in isolation is indicative of malignancy and that all features, clinical and histopathological, should be taken into account before reaching a diagnosis. However, two of these features are cited by major texts, and a lentiginous melanocytic growth pattern in an older patient (mean age, 48.6 years) in the absence of trauma is in our opinion supportive of at least in situ disease. The relative importance placed on these features may depend on whether one accepts the entity of dysplastic naevus, and here also we have a controversial entity with the problem of lack of reproducible histological features and disagreement over whether the lesion, if it exists, is a precursor to or risk marker of subsequent melanoma.

Melanocytic lesions comprise a heterogeneous group in which the biological behaviour of some of the more common entities is clearly understood. However, we believe it is essential for the less common entities (such as small cell melanoma) to be recognised and grouped with similar lesions so that accurate conclusions regarding their biological behaviour can be made; unless the entity that we have labelled “small cell melanoma” is clearly defined we will never collect the long term follow up data that will enable an assessment of its true biological potential. Until then, it is important that we all keep an open mind and in the words of the English philosopher Bertrand Russell who on being asked if he would be willing to die for his beliefs replied: “Of course not. After all, I may be wrong.”²

If “small cell melanoma” is the next Spitz naevus that’s OK by us.

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1 Blessing K, Grant JJH, Sanders DSA, *et al.* Small cell malignant melanoma: a variant of naevoid melanoma. Clinicopathological features and histological differential diagnosis. *J Clin Pathol* 2000;53:591–5.

2 Bertrand Russell (1872–1970), English philosopher, mathematician, essayist and social reformer. *The Times book of quotations*. Glasgow: Harper Collins, 2000.

Clear cell carcinoma of the ovary arising in a mucinous cystadenoma

I read with interest the recent case report by Drs Dutt and Berney “Clear cell carcinoma of the ovary arising in a mucinous cystadenoma”.¹ As these authors state, ovarian clear cell carcinomas often arise in endometriosis with a quoted incidence of associated pelvic endometriosis in 50–70% of cases and an incidence of endometriosis in the same ovary of 25%.² This is undoubtedly a gross underestimate of the association between ovarian

endometriosis and clear cell carcinoma because in many cases the tumour will overgrow and completely obliterate the endometriosis. However, my personal experience is that when ovarian clear cell carcinomas are extensively sampled (with special reference to cystic areas) the frequency of endometriosis in these neoplasms is substantially higher and indeed the origin of the tumour in an endometriotic cyst can be identified in most cases.

Drs Dutt and Berney might consider the possibility that the pre-existing cystic areas in their case, in fact, represent an ovarian endometriotic cyst with mucinous metaplasia. Figure 1 looks like the picture often seen in ovarian endometriotic cysts with mucinous metaplasia and fig 2 (right hand side) looks like the atypical changes sometimes seen in such cysts. The single cell lining of clear cells (fig 2, left hand side) is often seen in clear cell carcinomas arising in endometriotic cysts. Mucinous metaplasia can be extensive in ovarian endometriotic cysts³ and, in such instances, a diagnosis of mucinous cystadenoma may be considered. In addition, borderline mucinous tumours may arise in endometriotic cysts.^{3,4} Within the ovary a definitive diagnosis of endometriosis (especially an endometriotic cyst) is often difficult because there may be secondary changes in both the glandular and stromal elements. In particular, in endometriotic cysts, typical endometrial type stroma is often sparse or absent altogether and instead the stroma usually has a fibrous appearance.

There is evidence in the literature that many ovarian endometriotic cysts are, in fact, benign neoplasms and are not related to usual pelvic/abdominal endometriosis. Ovarian endometriotic cysts are often solitary and not associated with generalised pelvic/abdominal endometriosis. Studies, using X linked polymorphic markers, have demonstrated that ovarian endometriotic cysts may be monoclonal,^{5,6} supporting the hypothesis of a benign neoplasm, and DNA aneuploidy has been found in atypical areas.⁷ Because benign and borderline serous and mucinous cystadenomas are common but the corresponding endometrioid neoplasms are rare, it may be that endometriotic cysts with and without atypia correspond to benign and borderline endometrioid cystadenomas, respectively. This would provide an explanation for the coexistence of ovarian endometrioid and clear cell carcinomas with endometriosis.

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The authors reply

We are grateful to Dr McCluggage for his interest in our paper. We would like to reiterate our report that in this case there were no identifiable endometriotic components: the tumour appeared to be a classic benign mucinous cystadenoma.

He suggests that the mucinous areas might represent an endometriotic cyst with complete mucinous metaplasia. After extensive sampling (as reported), we found no areas of endometriosis. In fact, as we stated, there was a mucin filled multicystic area lined by typical picket fence mucinous cells. This area comprised one third of the total tumour, which was 24 cm in maximum diameter. This seems incompatible with an endometriotic origin. The benign mucinous areas in the tumour are illustrated (fig 1).



Figure 1 Multicystic area of tumour with benign mucinous epithelium, mucin within cysts, and an absence of endometrial stroma.

We entirely agree that clear cells forming a single cell lining are common in clear cell carcinomas arising in endometriotic cysts, but this represents simply a change to clear cell morphology, and cannot be taken to impute the derivation of the benign lesion.

Dr McCluggage suggests that endometriotic cysts can show a lack of endometrioid stroma or that they can be "fibrous". If a multicystic endometriotic cyst has global mucinous metaplasia and a complete lack of endometrioid stroma, then we can only say that in our eyes this would be taken to be a mucinous cystadenoma.

To suggest that a clear cell tumour always arises from an endometriotic cyst seems to be too didactic a viewpoint. Our case demonstrated that other pathogeneses may give rise to clear cell carcinoma.

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Rhabdoid phenotype in cutaneous squamous carcinoma: an earlier report!

We read with interest the case report by Mathers and O'Donnell on squamous carcinoma of the skin with a rhabdoid

phenotype.¹ The authors have indeed beautifully demonstrated the squamous histogenesis in their tumour. However, we are surprised by their statement that this is the "first case of cutaneous malignant rhabdoid tumour showing clear squamous histogenesis".

During their literature search, the authors appear to have missed our paper on a similar topic.² In 1996, we published a report of two squamous carcinomas with a rhabdoid phenotype. One of them was in the skin of an 85 year old man. The neoplasm had areas of conventional squamous carcinoma as well as large areas with a rhabdoid phenotype. The rhabdoid cells had diastase resistant periodic acid schiff material. The rhabdoid cells were positive for cytokeratin, epithelial antigen, and vimentin, but negative with antibodies to desmin, S-100 protein, and HMB45.

Our abstract clearly contained the words "skin", "squamous carcinoma", and "rhabdoid phenotype" and should have been picked up on a MEDLINE search.

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The authors reply

We thank Pai *et al* for their interest in our paper "Squamous carcinoma of the skin with a rhabdoid phenotype".¹ We apologise for our omission of their previous paper² in our review of the literature. However, we are pleased to hear that other authors have described rhabdoid differentiation within a squamous carcinoma of skin, as we feel this represents an important phenotype, which is predictive of a poor clinical outcome.

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Correction

Immunohistochemistry for MSH2 and MHL1: a method for identifying mismatch repair deficient colorectal cancer. *J Clin Pathol* 2001;54:484-7.

In the text the MSH2 and MHL1 genes were sometimes mistakenly written as MSH1 and MHL2, respectively. The authors apologise for this error.



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