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Title:

Additional loss of MSH2 and MSH6 expression in sporadic deficient mismatch repair colorectal cancer due to MLH1 promoter hypermethylation.

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Abstract:

Colorectal cancer (CRC) is common with 3% of cases associated with germline mutations in the mismatch repair pathway characteristic of Lynch syndrome (LS). The UK National Institute for Health and Care Excellence recommends screening for LS in all patients newly diagnosed with CRC, irrespective of age. The Yorkshire Cancer Research Bowel Cancer Improvement Programme includes a regional LS screening service for all new diagnoses of CRC. In the first 829 cases screened, 80 cases showed deficient mismatch repair (dMMR) including four cases showing areas with loss of expression of all four mismatch repair proteins by immunohistochemistry. The cases demonstrated diffuse MLH1 loss associated with BRAF mutations and MLH1 promoter hypermethylation in keeping with sporadic dMMR, with presumed additional double hit mutations in MSH2 +/- MSH6 rather than underlying LS. Recognition and accurate interpretation of this unusual phenotype is important to prevent unnecessary referrals to clinical genetics and associated patient anxiety.

BACKGROUND

Approximately 15% of colorectal cancers (CRC) are associated with deficient mismatch repair (dMMR) resulting in high-frequency microsatellite instability (MSI). The majority of dMMR cases are caused by sporadic hypermethylation of the MLH1 gene promoter region; however, around 3% of all CRC patients (20% of dMMR) contain a germline mutation in one of the MMR genes (MLH1, PMS2, MSH2 and MSH6) characteristic of Lynch syndrome (LS)[1]. The United Kingdom National Institute for Health and Care Excellence (NICE) guidance published in February 2017 recommends routine screening of all CRC patients at diagnosis[2]. Screening involves either four protein immunohistochemistry (IHC) or microsatellite instability (MSI) testing, followed by BRAF mutation and MLH1 promoter methylation analysis as appropriate in cases with MLH1 loss/MSI. Cases with MSH2, MSH6 or isolated PMS2 loss, or those with MLH1 loss with no evidence of a BRAF mutation or MLH1 promoter hypermethylation are recommended to undergo germline testing following appropriate counselling. Following IHC, most cases are straightforward to interpret, however, unusual patterns have been reported and incorrect interpretation could lead to unnecessary referrals for germline testing with associated patient/family anxiety and healthcare costs. Here we report four cases showing areas of additional loss of MSH2 and MSH6 on a background of sporadic MLH1 promoter hypermethylation identified in a large regional LS screening programme. In addition we have provided guidance for an approach to further testing and the clinicopathological interpretation of such patterns to ensure appropriate patient management.

METHODS

LS screening was made available through the Yorkshire Cancer Research Bowel Cancer Improvement Programme (YCR BCIP) to all 16 hospitals within the Yorkshire and the Humber region from May 2017. IHC for the MMR proteins was carried out as previously described on a DAKO Autostainer Link 48 using DAKO reagents (DAKO, Ely, UK)[3]. Loss of expression was defined as negative staining in tumour cell nuclei in the presence of adjacent positive internal controls (stromal cells, infiltrating lymphocytes or normal colonic crypts). For BRAF mutational testing, tumour rich cell-content was macro-dissected and DNA extracted using the QIAGEN QIAamp DNA Extraction Kit (QIAGEN, Manchester, UK). Analysis of mutation hotspots within

BRAF codon 600 (exon 15) was carried out by pyrosequencing as previously described[3]. Testing for MLH1 promoter methylation was carried out in Leeds Teaching Hospitals NHS Trust. Extracted DNA was treated with sodium bisulfate using the EpiTect Bisulfate Kit (QIAGEN, Manchester, UK) prior to undertaking amplification with primers specific to the MLH1 'C' region within the MLH1 promoter (QIAGEN, Manchester, UK). This product was sequenced using pyrosequencing and a percentage methylation value for each of the four CpG sites tested was produced. The average methylation across these sites was calculated and a result of >20% reported as methylated[4].

Further tissue blocks were requested from unusual cases and all slides were digitally scanned at 200x magnification using the Aperio XT system (Leica Biosystems, Vista, California). Using Aperio ImageScope, the total tumour area and areas of clonal loss were annotated on digital slides to calculate the percentage loss as a proportion of the total tumour area. Individual patient characteristics are summarised in Table 1.

RESULTS

By the 1st March 2018, 829 cases had been screened of which four (0.48%) showed an unusual loss of all four MMR proteins within regions of the tumour. None of these cases had undergone pre-operative treatment. Tumour morphology did not vary between the areas of discrepant staining.

In Case 1, IHC was initially performed on the diagnostic biopsy and subsequently on two blocks from the resection specimen. There was loss of MLH1/PMS2 expression in tumour cell nuclei across the entire tumour in all samples, with well-defined areas showing additional loss of staining of both MSH2 and MSH6 in the biopsy (71% of the tumour area) and one of the resection blocks (76% of the tumour area, figure 1). The other resection block showed normal expression of MSH2 and MSH6 throughout the tumour.

Case 2 consisted of a single resection block and showed similar features to case 1 with complete loss of expression of MLH1/PMS2 in the tumour, and a well-defined small area (5% of the total tumour) showing loss of expression of MSH2/MSH6. Additional blocks were not available for testing.

In Case 3, IHC was initially performed on a single resection block. The tumour showed loss of expression of MLH1/PMS2 throughout with small well-defined areas of combined MSH2/MSH6 loss (1% of the total tumour area) and other areas showing isolated MSH6 loss (40% of the total tumour area, figures 2 and 3). An additional block was received and demonstrated a slightly larger area of combined MSH2/MSH6 loss (8% of the total tumour area).

Case 4 consisted of diagnostic biopsies from synchronous ascending colon and rectal cancers. The rectal cancer showed strong nuclear staining for all four proteins throughout. In contrast, the ascending colon cancer showed complete loss of expression of all four MMR proteins (figure 4). No resection was performed and thus we were unable to confirm the presence of a null phenotype throughout the tumour.

All four cases contained a BRAF V600E mutation and all showed MLH1 promoter hypermethylation (Table 2).

DISCUSSION

We describe four cases with sporadic MLH1 deficient tumours showing additional loss of MSH2 and MSH6 in variable sized regions. Although a recent study from Pakistan identified 7 of 100 CRC patients with a null phenotype, similar findings in a large series have not been reported in Western populations[5]. To our knowledge only two other single case reports have detailed loss of expression of all four MMR proteins; one in a sporadic CRC and the other in a LS-associated CRC[6,7].

All four cases demonstrated complete MLH1 and PMS2 loss associated with a BRAF V600E mutation, which according to the NICE guidance are in keeping with sporadic dMMR rather than LS [2]. BRAF mutations are strongly associated with hypermethylation of the MLH1 gene promoter and a high prevalence is seen in sporadic CRC [1]. The presence of MLH1 promoter hypermethylation in all four cases provides further evidence for sporadic dMMR, although this would not have been routinely performed in straightforward cases of MLH1 loss according to the NICE guidance pathway. Such cases are well recognised to be associated with very high numbers of DNA mutations, therefore secondary double hit somatic mutations

in MSH2 and/or MSH6 may occur at varying points in tumour evolution. This mechanism has recently been described in a case of null phenotype due to co-existing sporadic MLH1 promoter methylation and double somatic truncating mutations in MSH2[7]. This would fit with the pattern of staining noted in Cases 1 and 2. The separate areas of MSH2/MSH6 loss and isolated MSH6 loss in Case 3 likely represents double somatic mutations in both MSH2 and MSH6 in different clonal regions. To our knowledge, this has not been previously described in the literature.

A reassuring feature in Cases 1, 2 and 3 is the clonal nature of MSH2 and MSH6 loss. This supports a subsequent double hit somatic mutation at a later stage of cancer development due to the high mutational load associated with microsatellite instability rather than LS. If these changes represent initiating events as seen in LS, they should be present throughout the entire tumour. Although somatic mosaicism has been reported in a patient with LS, it is extremely rare and to our knowledge only one single case report has described this finding[8]. The null expression observed in Case 4 was present throughout the entire submitted material, however, only a biopsy was available for testing and it is possible that areas of retained expression exist elsewhere. Unfortunately, the patient did not undergo resection for us to be able to determine this. The reassuring feature in this case is the presence of a synchronous rectal cancer with normal MMR. This would be very unusual in LS with previous studies showing matching MMR status in all LS-associated synchronous CRC[9,10].

BRAF mutations are very rarely seen in cases of LS-associated CRC[11]. In one case showing null expression of all four MMR proteins, the patient had a germline mutation in MSH2 associated with MLH1 promoter hypermethylation, but no BRAF mutation was identified[6]. The presence of a BRAF mutation is therefore a reassuring feature in null expression dMMR.

It is accepted that following the NICE guidance pathway will not identify all cases of LS. When determining the likely risk of LS, or other CRC predisposition syndromes, the presence of a strong family history should be taken into account if this is available and germline testing should still be considered in those patients with a clinical suspicion but who don't have an abnormal result following tumour screening.

Patient age at diagnosis can also be helpful, however, the presentation of LS can occur later in life; this is demonstrated in a screening study showing that 10 of 23 patients identified with LS were over the age of 50 years[12]. There is a difference in the mean age of diagnosis of LS dependent on the affected gene; patients with MLH1 and MSH2 mutations typically present with cancers earlier than patients with MSH6 and PMS2 mutations[12-14]. In all of our cases, the patients were aged between 76 and 83 years at the time of diagnosis, which along with the features above is further support for sporadic aetiology[1]

Other unusual staining patterns without a null expression phenotype have previously been described including the heterogeneous loss of MSH6 alongside loss of MLH1/PMS2 due to somatic mutations in coding exons of MSH6 in MLH1 and/or PMS2 deficient tumours[16-18]. It is important to note, that unlike our cases, MSH2 expression was maintained in these cases. In addition, the patchy or complete loss of MSH6 expression following chemo-radiation in mismatch-proficient tumours is well documented[16,17,19].

Unusual patterns of dMMR can create difficulties for histopathologists, specifically when it comes to whether or not to recommend consideration of germline testing. This is especially relevant when the patterns of expression do not fit within the common patterns described in the NICE guidance. We recommend that the staining pattern of each antibody is reported with areas of loss described as complete or partial. Loss of staining should always be confirmed as genuine by comparison to adjacent internal positive controls in the stromal cells and/or normal mucosa. Based on the four cases described, we recommend that cases showing complete loss of MLH1/PMS2 and additional clonal loss of MSH6 +/- MSH2 should undergo BRAF mutational analysis, followed by MLH1 promoter methylation analysis if BRAF is wild type. If a BRAF mutation or MLH1 promoter hypermethylation is present, the features are in keeping with sporadic CRC with secondary somatic events in MSH6 +/- MSH2, and a clinical genetics referral is not necessary. If the null expression pattern is observed throughout the whole tumour, this should be confirmed on resection material if initially performed on the biopsy. If a resection is not available or complete null expression is confirmed in the resection, we recommend a clinical genetics referral even in the presence of a BRAF mutation/MLH1 promoter

hypermethylation due to the possibility of a germline MSH2 mutation in association with sporadic MLH1 promoter hypermethylation. In all cases, the final decision whether or not to make a referral should be made by the treating clinical team who may be aware of independent risk factors for LS in the patient/family history and also whether the patient and/or their family consent to a referral being made.

CONCLUSION

Additional loss of MSH2 and MSH6 in MLH1 deficient CRC was observed in 0.48% of patients following LS screening in a large regional programme. In the four cases reported, all patients demonstrated features in keeping with sporadic dMMR rather than LS. Recognition of these unusual patterns of loss is important to facilitate the accurate histopathological reporting of dMMR and prevent unnecessary referrals to clinical genetics for counselling and consideration of germline testing. This will reduce the potential stress on patients and their families and also on increasingly precious healthcare resources.

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Competing Interests:

None declared.

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Figure legends

Figure 1. A. MSH2 immunohistochemistry in a resection block from Case 1 showing clonal loss in 76% of the total tumour area (x0.5 magnification). The area of associated MSH6 loss was complimentary to that of the MSH2 loss. **B.** Loss of MSH2 expression with adjacent positive stromal cells acting as an internal control (x20 magnification) **C.** Normal MSH2 expression in the remainder of the tumour and associated stromal cells (x20 magnification).

Figure 2. A. MSH2 immunohistochemistry in a resection block from Case 3 showing 1% clonal loss across two separate areas (x0.5 magnification). **B-C.** Areas of loss of MSH2 expression with staining in the adjacent stromal cells (x20 magnification).

Figure. 3. A. MSH6 immunohistochemistry in a resection block from Case 3 showing 40% clonal loss across multiple separate areas (x0.5 magnification). **B.** Isolated loss of MSH6 (29% of tumour) in tumour nuclei with positive stromal cells in the area corresponding to retained expression of MSH2 as seen in figure 2 (20x magnification) **C-D.** Complete loss of MSH6 (11%) in the areas of corresponding MSH2 loss as demonstrated in figure 2 (x20 magnification).

Figure. 4. Mismatch repair immunohistochemistry on biopsy blocks from the synchronous ascending colon and rectal cancers in Case 4 (x10 magnification). **A-D:** Loss of expression of all four mismatch repair proteins in the ascending colon tumour with adjacent stromal cell staining. This pattern was observed throughout the whole tumour in the biopsy. **E-H:** Retained expression of all four mismatch repair proteins in the rectal tumour.

Table 1. Clinicopathological data.

Case	1	2	3	4	
				Tumour 1	Tumour 2
Age (years)	78	81	83	76	
Gender	Female	Female	Female	Male	
Tumour site	Transverse colon	Caecum	Ascending colon	Ascending colon	Rectum
Specimen(s)	Biopsy	Resection	Resection	Biopsy	Biopsy
MLH1 IHC	Complete loss	Complete loss	Complete loss	Complete loss	Present
PMS2 IHC	Complete loss	Complete loss	Complete loss	Complete loss	Present
MSH2 IHC	Clonal loss	Clonal loss	Clonal loss	Complete loss	Present
MSH6 IHC	Clonal loss	Clonal loss	Clonal loss	Complete loss	Present

IHC = immunohistochemistry

Table 2. BRAF and MLH1 promoter methylation results.

Case	1	2	3	4
Specimen tested	Biopsy	Resection	Resection	Biopsy
BRAF status (mutation allele frequency)	V600E mutation (26.7%)	V600E mutation (26.4%)	V600E mutation (34.5%)	V600E mutation (47.9%)
MLH1 promoter methylation status (average methylation across four CpG sites)	Hypermethylated (64.0%)	Hypermethylated (42.6%)	Hypermethylated (68.3%)	Hypermethylated (41.5%)