Immunochemistry screening for Lynch syndrome in colorectal adenocarcinoma using an initial two antibody panel can replace a four antibody panel

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Abstract

Aim The current practice in immunochemistry staining for Lynch syndrome (LS) is to use a four-antibody panel, (MLH1, MSH2, MSH6, PMS2) to screen for the four Mismatch Repair (MMR) gene expressions involved.

We hypothesised that testing two antibodies (MSH6 and PMS2), followed by the other two only when there is loss of expression of the first two antibodies, would be equally effective as a four antibody panel in detecting LS. This hypothesis is based on the biochemical binding properties of the MMR proteins.

Methods We tested this hypothesis on a patient cohort consisting of all cases of colorectal cancer that were stained for MMR gene expression at Auckland City Hospital (Auckland, New Zealand) from the years 2000 to 2010 (inclusive), providing a series of 410 cases for this study. Exclusions were made based on heterogeneous staining pattern and unsatisfactory staining results on MSH6 and PMS2, which left n=400 included in the study.

Results The MMR gene protein stains were regarded as demonstrating loss of expression (LOE) when there was no uptake in the nucleus of the tumour cells, with a positive internal control. The results from our analysis supported our hypothesis. Seventy-four cases showed LOE of MSH6 or PMS2. One of them showed LOE of all four MMR proteins. For the remaining 326 cases, there was no LOE of all four MMR proteins.

Conclusion Our study gives further evidence that an initial two-antibody panel consisting of PMS2 and MSH6 would be as effective as a four-antibody panel in detecting DNA MMR gene protein LOE. This study has implications for significant cost cutting and improved efficiency in detection of DNA MMR gene protein LOE in LS.

Lynch syndrome (LS), also known as hereditary non polyposis colorectal cancer (HNPCC), is an inherited autosomal dominant condition predisposing to early onset colorectal adenocarcinoma.

Whilst environmental factors play a leading role in the aetiology of most colorectal cancers, inherited genetics are significant in between 15% and 30% of all colorectal cancers and approximately 5% of all colorectal cancers are due to an inherited syndrome.[1]

LS was first categorised by Professor Henry T Lynch in 1966 and is the most common of the inherited colon cancer susceptibilities, accounting for between 1% and 5% of all colorectal cancers.[1,2]

LS is inherited as a dominant disorder and affected individuals are at higher risk of developing colorectal cancer, endometrial cancer and various other types of aggressive cancers with rates quoted in most familial cancer registries of an approximate 80% lifetime risk for colorectal cancer.[2,3]

LS is caused by germline defects in at least one of a set of mismatch repair (MMR) genes in the mismatch repair system, namely MLH1, MSH2, MSH6 and PMS2.[1,4-8] Normal mismatch repair requires the function of several different genes products being co-ordinated. Loss of one or more of these MMR gene products leads to micro satellite instability (MSI) and cancer development.[4-5]

The role of the MMR system is to preserve genomic integrity and prevent replication errors by correcting base substitution mismatches and small insertion-deletion mismatches which are generated by errors in base pairing during DNA replication. Loss of MLH1 can also occur in the absence of germline mutations of the MMR gene, in cases of sporadic CRC. This is due to hyper-methylation of the MLH1 promoter and subsequent silencing of the gene. It is usually associated with BRAF gene mutation and may be seen in up to 15% of all CRC.[9] It can be identified by use of BRAF V600E mutation testing.

Of the above named MMR genes, germline mutations of MLH1 and/or MSH2 account for 80% - 90% of all cases of LS.[5,8] As such, the original immunohistochemistry (IH) panel used to screen for LS was a two-antibody panel of MLH1 and MSH2. This panel was later expanded to a four-antibody panel due to shortcomings with IH staining for MLH1.[10]

Namely it was found that whilst loss of MSH2 was very sensitive in detecting MSH2 defect carriers, some mutations of MLH1 still allowed weak positive staining of MLH1 and could lead to false negative results.[10] This is because up to one-third of MLH1 mutations are missense mutations results in functionally inactive but antigenically intact MLH1 mutant protein.

For such tumours, a false normal staining pattern with MLH will be observed. However, IH expression of its secondary partner PMS2 will be absent. The currently widely used four-antibody panel includes MLH1 and MSH2 as well as MSH6 and PMS2 and these additions have increased the sensitivity of the screening process.[11]

The biochemical binding properties of MMR proteins in forming functional heterodimer complexes can be extrapolated and used to further refine the current practice of using a four-antibody panel in testing for HNPCC. This is because MLH1 and PMS2 form a heterodimer complex, as do MSH2 and MSH6, in which MLH1 and MSH2 are obligatory partners and stabilise PMS2 and MSH6 (the minor partners).[11]

Loss of either obligatory partner leads to proteolytic degradation of the heterodimer complex and loss of both proteins, whilst the reverse is not true as the obligatory partners can bind with other minor MMR proteins (MLH1 with PMS1 or MLH3 and MSH2 with PMS3) and form stable heterodimer complexes.[12]

Therefore, mutation of MLH1 or MSH2 would result in loss of either MLH1/PMS2 or MSH2/MSH6 respectively. Conversely, mutation of PMS2 or MSH6 would not result in LOE of MSH1 or MSH2, respectively.

We hypothesise that staining for PMS2 and MSH6 alone will be sufficient to detect all cases of LS, and could replace routine screening with all four antibodies. Two recent papers, Shia et al[7] and Hall et al[8] have found the use of a panel consisting of just these two stains reduces costs whilst protecting the accuracy in HNPCC screening.

The aim of this study is to test this hypothesis by retrospectively applying it to the cases previously identified over an eleven year period in the Histopathology Laboratory of Auckland Hospital and if proven accurate to subsequently aid in changing current practice.

Methods and Materials

All cases of colorectal cancer diagnosed at or referred to Auckland City Hospital, and underwent IH staining for MMR gene protein expression over the 11 year period (2000-2010) were reviewed. The IH staining was performed in patients who satisfactorily met, at first, either the revised Amsterdam or Bethesda criteria, and later, the revised Bethesda criteria of 2004.[13,14] The upper age limit was modified to encompass all cases of MMR gene CRC at the Histopathology Laboratory of Auckland Hospital.

Sections of paraffin embedded tissue are cut at 4µm, and incubated for 30 minutes at 60°C. The slides are then stained using extended heat retrieval methods. Slides are incubated at high temperature for 90 minutes in citrate based buffer solution. All four primary antibodies are incubated for between 45 to 60 minutes. In recent years (2009-present) manual immunohistochemical staining has been replaced by automation using Ventana Ultra machines. Novocastra and Cellmarque are the primary antibodies of choice.

Immunohistochemistry staining results were reviewed initially looking for any loss of expression of MSH6 and PMS2. These stains are regarded as demonstrating LOE when there is no uptake in the nucleus of the tumour cells, with positive internal control. If either of these MMR gene proteins were lost then the results of MLH1 and MSH2 were also looked at. These results were then compared with the results found with all four stains interpreted together.

Ten cases show partial or unsatisfactory staining. These cases either involve PMS2/MSH6 (3 and 1 cases respectively), MLH1 with PMS2 LOE (2 cases) or MSH2 with MSH6 LOE (4 cases). Partial staining means weak or negative staining for MMR protein in the tumour cells, which may be either focal or diffuse, with strong staining of the stromal cells.

Unsatisfactory staining refers to negative staining with both tumour and stromal cells and satisfactory external positive control. These slides are repeated at least once, and also repeated on another tumour block. They are only included in this category if all of the slides show unsatisfactory staining. These cases are excluded from the study.

During the study period the four panel MMR staining has also been performed on adenomas, other primary cancers and old cases prior to the study period. These cases were excluded from the study.

Under the permission of the New Zealand Multi-region Ethics Committee, information was gathered on the patients and then all identifying marks removed. The patients were from the greater Auckland region. The patients included in the study ranged from 21 to 108 years old. The sites of cancer were various and included the entire colon.

Results

During the 11-year period, MMR staining according to the selection criteria was performed on 410 cases. Ten cases with partial/unsatisfactory staining result were excluded, leaving 400 cases in the study.

Seventy-four cases showed LOE of either MSH6 or PMS2 (18.5%). Among them 25 cases showed LOE of MSH6 (6% of total cases and 34% of cases with LOE) and 50 showed LOE of PMS2 (12% of the total and 67.5% of the cases with LOE). One of them showed LOE of both MSH6 and PMS2.

On subsequent review of the 25 cases with MSH6 LOE, 13 also showed concurrent LOE of MSH2 (52%). Of the 50 cases with PMS2 LOE, 41 showed concurrent LOE of MLH1 (82%). The case with LOE for both MSH6 and PMS has also lost MLH1 and MSH2. After reviewing all 400 cases there was no LOE of either MLH1 or MLH2 in isolation.

Table 1 reflects the pattern in which the expression was lost. In one sample expression of all four genes was lost, whereas the other 73 samples had loss of either the minor constituents (MSH6 and PMS2), or in some cases the major constituents as well. There was no isolated loss of the major constituents.

Table 1. Pattern with which MMR gene loss of expression was demonstrated

Loss of expression (LOE)	n
MSH6 only	12
MSH2 only	0
MSH6 + MSH2 only	12
PMS2 only	9
MLH1 only	0
PMS2 + MLH1 only	40
All four stains	1

Discussion

Our study further supports the evidence put forward by two separate recent studies from the USA[7] and Australia.[8] Shia et al[7] tested 232 tumours, and found LOE of at least 1 MMR gene in 70 cases (26%). Hall et al[8] tested 344 tumours with LOE in 104 cases (30%). Our study looked at 400 cases, with an 18.5% positive rate of 74 cases.

The lower positive rate is probably due to inclusion of a higher age group, as opposed to the revised Bethesda criteria of 50. In these three studies, with a total number of almost 1000 cases, the staining results indicate that the two initial stains (PMS2/MSH6) are equally as specific to diagnose LS when compared to all four stains. There is no LOE of MLH1/MSH6 without concomitant LOE of PMS2/MSH6 respectively. In our study, there is one cases of LOE of all 4 genes. In the series by Hall et al.[8] there are two cases of triple LOE for MSH1/PMS2/MSH6.

Shia[7] has reviewed four other series of MMR staining studies, which included a total of 1704 cases CRC, with LOE of at least one IH stain shown in 294 cases. There were two cases in one of the series[15] that showed loss of MLH1, but not PMS2. In another series from Australia, there are 235 MMR deficient cases from an unknown number of unselected CRC.

Ten cases show either isolated MLH1 loss (8), MSH2 loss (1) or both MLH1/MSH2 loss (1). However, all these 10 cases were confirmed not to be LS by germline mutation analysis.[16] It is likely that the two cases from the former series were also non-LS, although there was insufficient information to confirm.

Patients identified as having LOE of either of the two initial stains or partial or unsatisfactory staining patterns should be tested and worked up for the possibility of

LS. Hall et al[8] has proposed a strategy for germline mutation testing with either or both PMS2/MSH6 deficiency. With PMS2 loss, the suggested next step was to exclude CpG island methylator phenotype by BRAF V600E mutation analysis.

Mutated V600E indicate somatic methylation causing silencing of the MLH1, and excluded LS. Otherwise germline MLH1 mutation testing, and if negative, PMS2 germline mutation testing should be performed. In cases of MSH6 loss, germline MSH2 mutation should be checked for and if negative MSH6 mutation testing should be performed.

Ten cases in our study show partial or unsatisfactory staining in at least one of the four IH stains. The partial staining pattern may indicate mutation that cause partial or weak staining, and have been particularly implicated in MLH1.[12] It may also be seen in mutation negative and microsatellite-stable cases, and may be related to old paraffin blocks and quality of tissue preservation.[17]

Unsatisfactory staining pattern has also been well documented. They are best treated and tested as cases with loss of expression of the corresponding gene.[7,18] However, these cases with partial or unsatisfactory staining do not affect the validity of this study because they do not occur in isolation for the staining of the major constituents of the mismatch repair protein heterodimers. All this cases would have been discovered with the staining for the minor constituents (MSH6/PMS2), and further studies can be performed to determine whether it is a case of LS.

In a study conducted in 2008, Hampel et al[15] illustrated that when screening CRC patients for LS, IH staining is almost equally sensitive as microsatellite instability testing and is more readily available and helps to direct genetic testing as it identifies which of the MMR genes are abnormal. It also found that limiting tumour analysis to only those that fulfilled the Bethesda criteria would fail to identify 28% of cases of Lynch syndrome.

Colorectal cancer is the second most common registered malignancy to the New Zealand Cancer Registry.[19] Screening is an important tool in reducing colorectal cancer incidence and mortality in those most at risk and more frequent screening is required in those with proven LS due to their greatly increased lifetime risk of developing a colorectal cancer.[20]

Confirmation of LS cases may affect patient management, allow identification of at risk family members, who are in need of raised colonoscopic surveillance, and conversely to allow reassurance and avoid over-screening those at lower risk.[1]

In the past 10 years, MMR staining was limited to patients satisfying the aforementioned criteria. There has been a trend towards an increasingly liberal staining, which can at times include staining for all CRC cases that are detected. The policy in some hospitals is to do MMR testing on patients less than 50 years old and those with a strong family history.

The study by Hampel et al[15] illustrated that screening only patients younger than 50, or according to the revised Bethesda criteria, would fail to identify 28% of cases of LS. Their group also commented that it will become more difficult to identify patients with LS on the basis of family history of CRC in the future because of a) decreases in family size and b) increasing usage of screening via colonoscopy which could prevent

many CRCs through the removal of pre-cancerous polyps. This group suggested comprehensive screening for LS among all CRC patients. This view was echoed in a letter by a group from Austria[21] who cited that none of their 700 CRC patients (and only one of the 153 LS patients reported by Hampel et al[15]) had been referred by a clinician for genetic testing.

In the series by Hampel et al[15] it was found that for each pro-band there was on average three additional family members who carried MMR gene mutations. The potential benefit of increasing the IH screening for CRC in these patients is significant. We propose that a simplified initial MMR staining, utilising the discussed two stain panel, would allow the same resource to be used to screen for almost twice the number of CRC patients without additional cost, thus increasing the diagnostic yield of IH in LS.

In New Zealand, the NZCR recorded 3002 new cases of CRC in 2011.[22] If comprehensive screening for LS with IH staining was applied to all cases as an initial procedure, using two stains instead of four stains, this would reduce resource use by almost 6000 IH stains annually, without compromising the yield of LS cases.

The change in the staining strategy would dramatically reduce the number of patients who progress to a four-antibody panel as less than 5% of these CRC patients would require testing with all four antibodies. In turn this would save on laboratory costs, costs of manpower and most importantly save patients undergoing unnecessary tests.

Conclusion

Our study further reinforces previous reports from the USA and Australia which suggest that immunohistochemical staining for mismatch repair gene loss of expression can be successfully limited to two of the four stains currently used at the initial stage to detect Lynch syndrome. There are no false negatives by this approach, in which MSH6/PMS2 do not show loss of expression.

This proposed screening panel carries a significant benefit in cost and manpower saving in staining, could allow for greater screening without increased cost and more importantly comes without loss of sensitivity in detecting cases of Lynch syndrome.

Competing interests: Nil.

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