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Research Article

# Universal Immunohistochemistry Screening for Lynch Syndrome: Real World Lessons from an Academic Institutional Experience

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## Abstract

**Background and Aims:** Lynch Syndrome (LS) is the most common inherited form of Colorectal Cancer (CRC). While current guidelines recommend Universal Molecular Screening (US) of CRCs, the effects of this in clinical practice remains unknown.

**Methods:** Data on adult patients with surgical CRC resection between January 1, 2010 and December 31, 2014 were obtained. Previously diagnosed hereditary cancers, secondary colonic cancers and inflammatory bowel disease associated CRC were excluded.

**Results:** 455 patients were included with mean age 62 years; 74.7% (n=340) underwent US with Immunohistochemistry (IHC). Distal left sided CRCs were less likely to undergo IHC (22.6%, p<0.001) when compared to all other locations. Among those tested with IHC, 17.9% (n=61) had MMR loss; rates of prior personal or family history of uterine or colon cancer were similar regardless of MMR status. 47 (13.8%) patients had IHC consistent with LS of which less than half (47%) were referred for GC. Patients <50 years of age were more likely to receive GC (OR 4.30, 95% CI 2.17-8.52, p<0.001), even in the presence of negative IHC screens (OR 13.23, 95% CI 4.43-39.55, p<0.001). Confirmatory BRAF mutation testing for MLH1/PMS2 loss greatly improved accuracy of identifying LS.

**Conclusions:** A consistent approach to US of CRCs is imperative given that patients with loss of MMR were similar clinically to those with preserved MMR. Distal left sided CRCs should undergo adequate pre-treatment biopsy sampling to allow for US given the barriers to IHC in this location following radiation treatment and resection.

## Study Highlights

What is current knowledge:

1. Lynch Syndrome (LS) is the most common inherited form of Colorectal Cancer (CRC)
2. Current guidelines recommend Universal Molecular Screening (US) of CRCs utilizing immunohistochemistry
3. The effect of US with IHC in clinical practice remains unknown

What is new here:

- Patients with loss of Mismatch Repair (MMR) were similar clinically to those with preserved MMR.
- Distal left sided CRCs were less likely to undergo IHC
- Distal left sided CRCs should undergo aggressive pre-treatment biopsy sampling given the high rates of LS diagnosed in this location.

## Abbreviations

| | | |
|---|---|---|
| LS | : | Lynch Syndrome |
| CRC | : | Colorectal Cancer |

US : Universal Screening

MMR : Mismatch Repair

IHC : Immunohistochemistry

**Keywords:** Colorectal Cancer; Genetics; Gastroenterology; Pathology; Mismatch Repair

## Introduction

Lynch Syndrome (LS) is an autosomal dominant disease that underlies approximately 2-5% of all colorectal cancers (CRCs) [1], making it the most common syndrome of inherited CRCs [2]. LS is defined by a germline mutation in any one of the four Mismatch Repair (MMR) genes: PMS2, MLH1, MSH2, or MSH6, or by mutation in the EPCAM gene, causing loss of MSH2 expression. PMS1 and MLH2 form a dimer, as do MSH2 and MSH6, and germline mutation in either gene of the dimer often causes effective loss of functionality of both proteins [1,3]. Loss of MMR protein function results in microsatellite instability (MSI), where small repetitive DNA sequences are not corrected during replication leading to gene mutations [4]. MMR deficiency manifests clinically as early cancer development, and LS is characterized by greatly increased risk of CRC and endometrial carcinoma, as well as increased susceptibility to bladder, ovarian, biliary, and gastric cancers, among others [5].

In the case of CRC, Lynch syndrome patients often develop cancer before age 50, the standard age of initiation of population based screening. It is imperative to correctly identify LS patients among the general CRC population because of implications for screening, surgical treatment, and family planning. Significant evidence suggests that Lynch Syndrome is under diagnosed in the general population [6]. Previously, potential LS patients have been identified for molecular testing using the clinical Amsterdam [7] or Revised Bethesda [8] criteria. While the Amsterdam II criteria, based on clinical assessment and family history, is highly specific, its sensitivity is far less than 50%. The more lenient Bethesda criteria have sensitivities bordering on 75%, but have been found to be difficult to implement in clinical practice [9,10]. Likewise, computational models such as MMR predict and MMR pro, while more sensitive and specific than clinical assessments, suffer from improper and incomplete usage [11,12].

In recent years, universal molecular screening of CRC pathology samples for possible Lynch Syndrome has gained traction. Among the notable early groups to advocate for universal screening was the Evaluation of Genomic Application in Practice and Prevention, in 2009 [13]. Molecular screening typically refers to testing of tumor tissue pathology sections after resection for Microsatellite Instability (MSI) or for loss of MMR with Immunohistochemistry (IHC). MSI testing utilizes molecular testing of formalin-fixed tissue specimens and can identify the presence of significant microsatellite instability with high sensitivity (60-90%) and specificity (85-90%) standards [14]. Immunohistochemistry (IHC) staining also uses formalin-fixed tissue in pathological specimens, instead targeting the specific MMR proteins with immunofluorescent staining [15]. Like MSI, IHC has been shown to be superior to clinical or computer modeling to identify patients with possible Lynch Syndrome, with sensitivity and specificity each approaching 90% [14]. In addition, IHC has an advantage in that a positive test suggests which MMR protein is absent, guiding subsequent germline testing [2,16]. Overall, several studies have proposed that universal screening of resected CRCs is a cost effective approach to identifying LS patients [17,18].

Most recently, 2015 AGA guidelines [19] suggest that all resected CRCs should be screened for possible LS with either MSI or IHC molecular testing. Furthermore, [11], they recommend patients with abnormal IHC results be recommended for genetic counselling and germline testing for LS. In addition, studies have demonstrated that while sporadic cancers often develop MSI by hypermethylation of the hMLH1 gene, such a phenomenon is uncommonly found in LS [20-22]. This sporadic development, however, can mimic LS on IHC screening with loss of MLH1/PMS2 expression. The new AGA recommendations reflect this development, suggesting that MLH1 absence on IHC be followed with testing for hypermethylation of MLH1 and/or a BRAF mutation before referral to counselling [13,20,23].

Few studies have looked at the efficacy of such a strategy in clinical practice, although it has been noted previously that effective implementation would require extensive multidisciplinary contributions. Some studies have examined universal screening for a subset of patients, such as those under 50 years old or with right sided primary tumors [15]. Others have excluded older patients with no documented family history of CRC, even in the case of a positive screen [24]. In 2009, our University implemented a US policy with IHC for all CRC patients. Although the true efficacy of Immunohistochemistry versus Microsatellite Instability testing has not been examined in clinical practice, it has been postulated that IHC testing is more feasible and cost effective for a standard pathology laboratory, guiding our decision. We present our institutional experience with this IHC universal screening protocol.

## Methods

### Time Course and Setting

Our study is a retrospective cohort study conducted at the University of Virginia Medical Center between 2010 and 2014. In 2010, a new policy was implemented whereby all patients with resected colorectal cancers would receive IHC screening for loss of MMR proteins. The ordering of the IHC panel was delegated to the pathologist(s) assigned to the case and who provided the official histopathologic assessment of the resection specimens. The

results of the IHC screening were included as an addendum to the surgical pathology report, which were faxed to both the surgeon of record and the genetic counselor assigned to the gastrointestinal surgery service. The majority of addendum reports were available for review prior to the surgical follow up appointment by the attending surgeon.

In follow up, the surgeon is tasked with covering all pertinent pathologic findings with the patient, including any positive results of the IHC staining. Given a positive result, the surgeon would question the patient further on family history and discuss the possibility of genetic cancer, after which she would refer the patient to appropriate genetic counseling. A genetic counselor with specific knowledge of Lynch Syndrome and our testing protocol was made available to meet with these patients, elucidate further family and clinical history, and recommend appropriate germline testing.

### Molecular Screening with IHC

The IHC testing was performed in the CLIA-approved Medical Laboratories of the University of Virginia Hospital. During the study period the majority of IHC stains were performed on an automated platform (Autostainer, Dako Corp.) using EnvisionTM Dual Link (Dako) followed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Four-micron tissue sections were subjected to heat antigen retrieval at pH 9. Antibodies used were as follows: MLH1 (Cat. # 550838, BD Biosciences), PMS2 (Cat. # 556415, BD Biosciences), MSH2 (Cat. # CM219C, Biocare Medical), MSH6 (Cat. # 610919, BD Biosciences). Appropriate positive and negative controls were included for each IHC assay run, and for each case, respectively. The majority of IHC assays were interpreted by a single UVA staff pathologist with specialized expertise in the interpretation of these assays.

### Patient Selection

All relevant patients for this study were identified using the University of Virginia Clinical Data Repository (UVA-CDR). The UVA-CDR was queried for all patients receiving surgical resection of a CRC since implementation of the US policy. All patients with a primary colonic adenocarcinoma, regardless of age, site of diagnosis, or clinical presentation were included in the study. Only those patients with previously diagnosed hereditary cancers such Familial Adenomatous Polyposis or colonic cancers secondary from another site were excluded. In addition, patients receiving cancer resections with previously diagnosed Inflammatory Bowel Disease (IBD) were excluded.

### Data Collection and Analysis

The medical records of identified patients were reviewed thoroughly for multiple factors. They were classified based on date of birth, gender, location of tumor, surgical date, and status as living or dead. In addition, we determined whether they received IHC staining, appropriate surgical follow up and when, discussion of genetic cancer, and appropriate referral to genetic counseling. Finally, we reviewed their records for elements of their histories common among patients with Lynch Syndrome, including a family or personal history of colon or uterine cancer (and whether such a history was taken).

For patients with positive screens for loss of MMR, we noted which protein or proteins were lost and for those with MLH1/PMS2 loss, whether confirmatory BRAF testing was undertaken. While BRAF testing was not widely accepted as an important next step in those patients with MLH1/PMS2 loss on staining early in the study, it had become widely used by the end of the study period. Primary endpoints included successful IHC staining, and for those with MMR loss, surgical follow-up and discussion of Lynch Syndrome with appropriate referral to genetic counseling.

All Data Analysis was conducted using Microsoft Excel, and included Student's t-testing and Chi-Square testing with two-tailed p values. Graphs were made using GraphPad Prism. Permission for this study was obtained from the Institutional Review Board at the University of Virginia. In compliance with the IRB board, patients were not contacted with results regardless of the prior outcome of their treatment and screening.

## Results

With the above exclusion criteria applied, 455 patients with primary colorectal adenocarcinoma underwent surgical removal at our institution during the study period. The median patient age was 62, 243 (52.3%) were male, and 394 (86.6%) had a family and personal cancer history recorded prior to surgery or on follow up. Of these patients, 26% had family histories of colon or uterine cancer, and 15 of the 394 (3.8%) had previously had either colon or uterine cancer personally. Of the 455 patients in this study, 340 received IHC staining and interpretation, and 61 (13.4%) had positive screens for loss of MMR. The characteristics of patients who received or did not receive screening are found in Table 1.

| | Got IHC Staining n=340 | Not IHC Stained n=115 | p Value | Loss of MMR by IHC n=61 | No Loss of MMR by IHC n=279 | P Value | All Patients n=455 |
|---|---|---|---|---|---|---|---|
| **Average Age** | 61.9 | 62.3 | 0.78 | 64.2 | 61.4 | 0.139 | 62.0 |
| **Family History Taken** | 90% | 76.2% | 0.0007 | 90.2% | 90.0% | 0.970 | 394 (86.6%) |
| **Family History Colon/Uterine Cancer** | 32.7% | 20.2% | 0.031 | 43.6% | 30.2% | 0.053 | 118 (29.9%) |
| **Previous Cancer History** | 17.6% | 12.2% | 0.19 | 26.2% | 15.8% | 0.052 | 74 (16.3%) |
| **Previous Colon Cancer** | 8 (2.3%) | 2 (1.7%) | 0.99 | 3 (4.9%) | 5 (1.8%) | 0.145 | 10 (2.2%) |
| **Previous Uterine Cancer** | 5 (1.5%) | 1 (0.9%) | 0.99 | 1 (2.5%) | 4 (2.6%) | 0.904 | 6 (1.3%) |

**Table 1: Clinicopathologic parameters.** There was a statistically significant difference in the number of patients who had a family history taken and the percentage of these with a family history of colon or uterine cancer, in general the patient populations that did or did not receive IHC screening, or did or did not have loss of MMR on screening were not easily distinguishable.

Regardless of outcome of IHC screening, patients aged 50 or lower were almost four times as likely as those older than 50 to have the possibility of underlying genetic predisposition to cancer discussed with them (45.1 vs 17.2%, Odds Ratio 3.95, 95% CI 2.37 to 6.60, p<1E-7) and to receive genetic counseling (22.0 vs 5.6%, OR 4.30, 95% CI 2.17 to 8.52, p<1E-5). This phenomenon was actually most striking in patients with negative IHC screens, as those under 50 were thirteen times more likely to be referred for genetic counseling (OR 13.23, 95% CI 4.43 to 39.55, p<1E-9). Even among patients with a positive screen for MMR loss, the median age for a patient referred to counseling was 55.1±3.1 while that of a patient not referred to counseling was 70±2.2 (p=0.0002). This trend existed despite the fact that the median age of patients with a Lynch concerning MMR loss was 67, and the median age of patients with any non-MLH1/PMS2 loss was 57.

In addition, patients reporting a family history of colorectal or uterine cancer were more likely to receive discussion about such an underlying genetic predisposition (39.8 vs 17.0%, p<1E-5) and to be referred to genetic counseling (22.0 vs 4.7%, p<1E-6). Although patients with MMR loss were more likely to report a family history of uterine or colon cancer, it was not significant (p=0.052), even when considering that many of these patients were referred to genetic counseling and likely had a much more thorough family history queried.

In patients with positive screens for loss of MMR by IHC (Figure 1), 37 (60.6%) were found to have loss of MLH1 and PMS2. Among the remaining 24 patients with MMR loss on IHC screen, 7 stains demonstrated loss of MSH2 and MSH6, 9 demonstrated losses of MSH6, and 4 demonstrated losses of PMS2. Confirmatory BRAF testing was conducted on 13 of the 37 patients with loss of MLH1/PMS2. Of note, all but one of the confirmatory BRAF mutation tests were conducted in the second half of the study, with 9/13 (69%) conducted in 2014. In the second half of the study period, encompassing 2013 and 2014, 12 of the 14 patients with MLH1/PMS2 loss received confirmatory testing, as opposed to 1 of 23 between 2010 and 2012. Of the 13 patients who received confirmatory testing, 11 were found to have BRAF mutations. Four patients with loss of MMR on IHC staining died before surgical follow up, and while it is unknown whether their next of kin was notified of their MMR status, it is unlikely that any further evaluation was done.

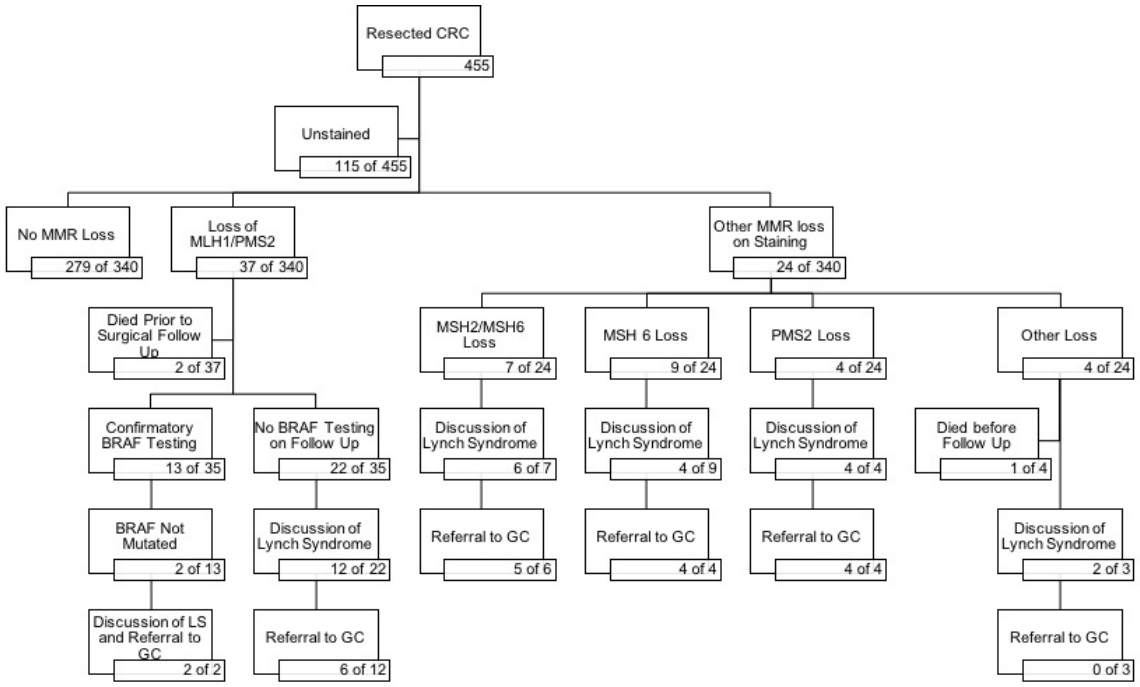
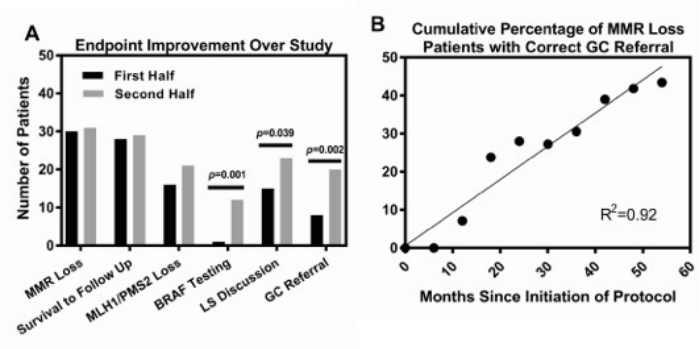



**Figure 1:** Flowchart representation of patient outcomes in this study by results of IHC staining. Patients were considered appropriate for LS discussion and GC referral if they had any loss of MMR, except those patients with BRAF mutation found on reflex testing of MLH1/PMS2 loss tumors.

In all, 47 of 455 patients in this study were found to have results concerning for Lynch Syndrome. For the purposes of this analysis, this includes those patients with loss of MMR proteins on IHC staining who survived to surgical follow up and were not found to have the somatic BRAF mutation. It includes patients with loss of MLH1/PMS2 who did not receive BRAF follow up testing, as confirmatory testing was not commonly executed during early stages of the study before a more structured follow up protocol was established.

The 47 patients all received surgical follow up and discussion of pathology results at a median of 51 days after surgery, with 30 (63.8%) instances of genetic cancer discussion. Of these 30 patients, 20 (67%) were referred to genetic counseling.

Over the course of the study, marked improvement was seen in referral patterns to genetic counseling if indicated. No significant difference was seen between the early and late parts of the study in taking of family histories, scheduling and achieving surgical follow up, or percentage of tumors stained for MMR loss. However, compared to the first half, patients in the second half of the study with MMR loss on staining were significantly more likely to have a discussion with the surgeon about Lynch Syndrome on surgical follow-up (54 vs. 79%, p=0.04) and to receive appropriate referral to genetic counseling per AGA recommendations (29 vs. 69%, p=0.002) (Figures 2A,B).




**Figures 2(A-B): A)** Representation of key endpoints from beginning to midpoint (n=228) and midpoint to end (n=227). A similar number of patients were found to have MMR loss, and to have survived to follow up, but a significantly higher percentage of the patients with MMR loss were correctly managed. **B)** Over the course of the study, the cumulative number of patients correctly referred to Genetic Counseling increased in a nearly linear fashion as visualized in six month increments.

Significant variance in IHC staining for loss of MMR was seen based on tumor location (Figure 3). In all, 115 resected tumors were not stained for loss of MMR. Tumors in the distal left colon (sigmoid colon, rectosigmoid colon, and rectum) accounted for 89 such cases (Figure 4), and were significantly less likely to be stained for IHC detection of MMR loss (Odds ratio 2.01; 95%

CI 1.24-3.24; p<1E-4) than those elsewhere in the colon, where only 26 of 181 (14.3%) were not stained for any reason. In 57 (64%) of these distal left colon tumors, no residual adenocarcinoma was found on pathology, mitigating the possibility of staining for MMR loss. In contrast, 8 of 26 (31%) of tumors located elsewhere were not assayed due to lack of residual tumor. Tumors in the distal colon were far more likely to be not assayed due to lack of residual tumor than those located elsewhere (Odds Ratio 5.59; 95% CI 2.60 to 12.02; p<0.0001). In the remaining patients who failed to receive US with IHC, reasons included 25 cases where the tumor was described as being exceptionally "low grade" and 25 cases where the lack of IHC results was entirely unexplained, suggesting a possible failure of the protocol being executed.

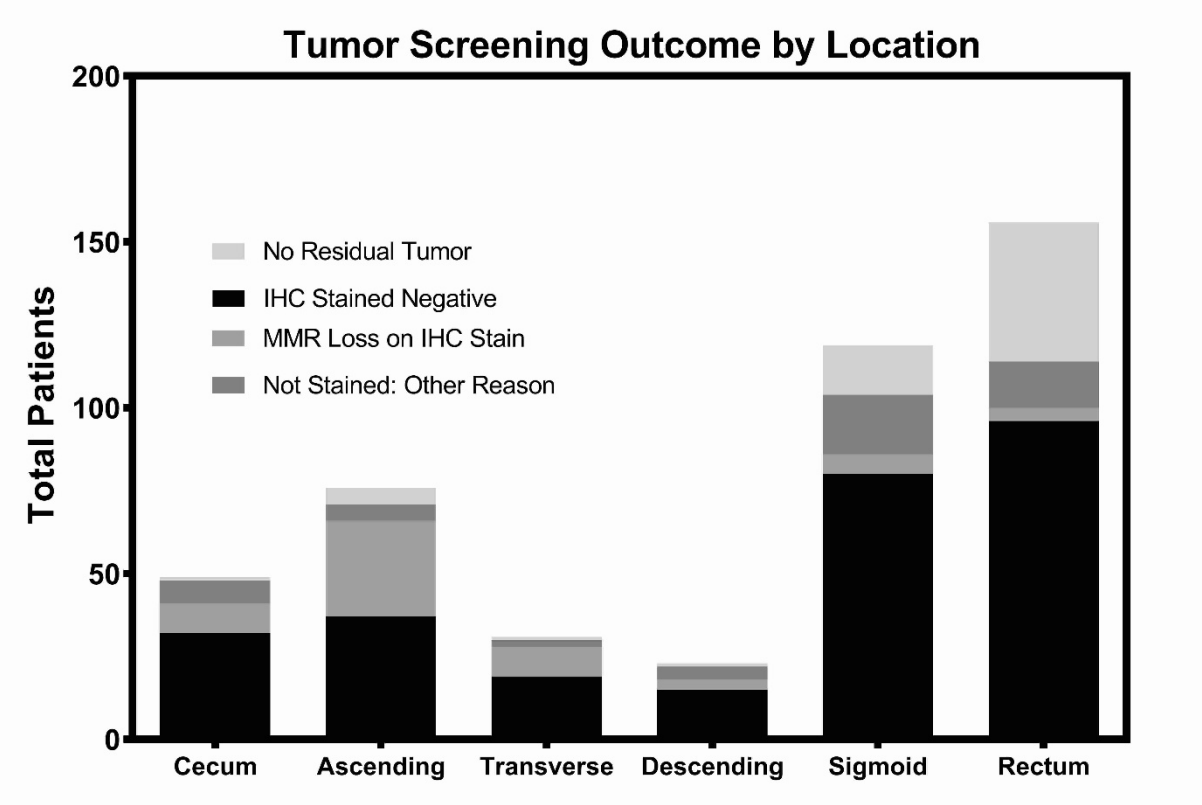



**Figure 3:** The outcome of IHC screening for LS was highly variable based on the location of the primary tumor. Distal tumors (sigmoid, rectum) were the most common overall, and were also the least likely to be successfully screened. Conversely, more proximal tumors, especially left sided ones, were more likely to demonstrate MMR loss on screening.

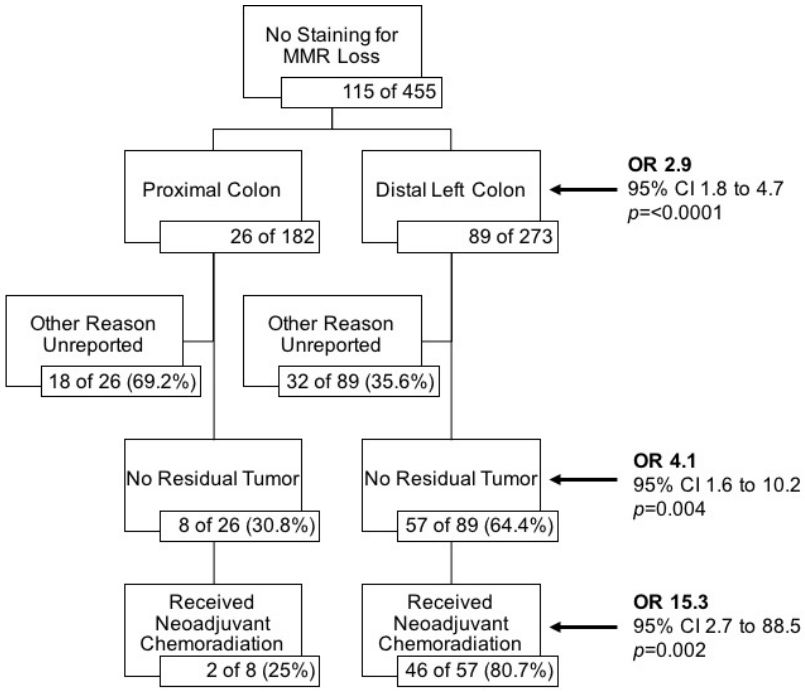



**Figure 4:** Patients with Distal Left Colon CRCs were significantly more likely to be unscreened for any reason by IHC. Even among specimens that were unscreened for MMR loss, patients with Distal Left Colon CRC were more likely to have no residual tumor available for staining, and to have received neoadjuvant chemoradiation therapy. Lack of staining for any other reason was not significantly different between the two subsets of tumor locations.

Patients with proximal tumor locations (Cecal, Ascending, Transverse, or Descending colon) who received staining had loss of MMR found in 50 of 181 instances (27.62%), while distal tumors had loss of MMR found in only 10 of 273 (3.67%) of stained tumors (Odds Ratio 10.0, 95% CI 4.93 to 20.42). While patients with MMR loss in the distal colon accounted for only 16.4% of all MMR loss found, they included 7 patients with total or complete loss of MSH6, 2 with total loss of MSH2/MSH6, and 1 with lone loss of PMS2, with no MLH1/PMS2 loss found. The median age in these patients was 54 and 4/10 had significant family history of colon or uterine cancer.

## Discussion

This study confirms the utility of universal screening of CRCs for detection of patients with possible Lynch Syndrome in clinical practice, as well as some of the shortcomings therein. Several studies have suggested that Universal Screening improves the rate of detection of Lynch Syndrome over more traditional screening methods. Recent guidelines reflect this thought, suggesting that all resected CRC should undergo molecular analysis for loss of MMR. Patients with loss of MMR did not have a markedly different demographic, clinical, or pathological profile from patients without this loss. This demonstrates the necessity of molecular screening to capture a significant number of these LS individuals. While many patients with MMR loss had easily identifiable traits suggesting hereditary cancer, such as family history or personal CRC or uterine cancer history and age under 50, only 9 of the 61 patients with MMR loss on staining had even two of the three.

While not specifically designed to evaluate history taking, extensive variance existed in the timing and documentation of family history. Such a criterion is extremely difficult in clinical practice, and the nearly universal implementation of electronic medical records and subspecialty referrals have the potential to make it uncertain where in the record to find such information. Universal Screening provides both improved detection rates over and back up for traditional history-based screening methods.

The 115 patients who failed to receive IHC staining and screening provide an interesting insight into the efficacy of tissue based screening in practice. These patients accounted for approxi-

mately 1 in 4 total patients in the study, but were heavily weighted towards those with distal CRC. Among these cases, the majority of failed screens were related to lack of residual adenocarcinoma found in the tumor, precluding IHC staining. The likely root cause of such a failure is the use of neoadjuvant chemotherapy for patients with tumors in the distal left colon, as opposed to tumors in more proximal colon. In fact, many pathology reports specifically noted a total chemotherapeutic response in these patients.

Interestingly, all of the MMR loss found in the distal left colon was of the Lynch suggestive variety; thus it is imperative to improve the yield of molecular screening in these patients. One possibility to mitigate the loss of viable tumor before resection would be to take extra biopsies of the tumor before initiation of neoadjuvant chemoradiation. In fact, it appears that neoadjuvant chemotherapy or radiation can cause acquired loss of MSH6 expression in CRC, even in patients with sporadic tumors with preserved staining in pretreatment biopsies [26]. Although a small study, it demonstrated that in all 9 cases of pretreated tumors and partial loss of MSH6, staining was preserved in the pretreatment biopsy. However, the single patient with complete MSH6 loss in the treated specimen also had loss in the pretreatment specimen and a likely germline mutation. Therefore, we suggest that all patients destined to undergo neoadjuvant therapy be screened from pre-treatment biopsies in which viable tumor tissue is found.

Surgical specimens, in general, are not without their shortcomings when testing with IHC for MMR loss [27,28]. As one study notes, IHC is very sensitive to correct fixation of tissue, which can prove difficult in large surgical specimens where there might be a long delay in the operating room without specimen fixation [29]. Current guidelines make no recommendation to proactively or retroactively test biopsy samples, despite evidence that CRC biopsies have similar if not better yield [30,31].

While tumors in the ascending and transverse colon, especially, were more likely to be found to have MMR loss on staining, the observed rate of MMR loss in the distal left colon (3.67%) suggests that neglecting to screen patients with distal tumors will surely result in missed LS cases. Among the ten patients with distal left colon tumor MMR loss in our study, there were 7 patients with MSH6 loss and 2 with MSH2/MSH6 loss, while only 4 had a substantial family history. This is in agreement with another study that looked at the location of origin of tumors in their Lynch Syndrome database who found 25% of all MSH6 deficient tumors originated in the rectum, which was much higher than all other MMR deficiency [25]. In their database, patients with MSH6 loss were also more likely to be of older age and with more sporadic appearing family histories.

The improvement in compliance with our US protocol over the course of the study is noteworthy. A previous study has examined how change in reporting protocol during a study improves compliance by giving earlier access to the pathology results to genetic counselors [24]. Improvement over the course of this study likely was related to more familiarity with our protocol and development of the infrastructure to execute among relevant faculty and staff. It demonstrates that there is a strong correlation between experience and functionality of a protocol. Continuity among colorectal surgeons, pathologists, and genetic counselors was crucial. Any institution implementing a universal screening protocol should consider continuity, training, and follow up evaluations as crucial aspects to successfully execute this type of universal screening.

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