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Original Research

Concurrent immunohistochemical testing of L1CAM and MMR proteins adds value in risk stratification of endometrial cancer: a proof of concept

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Objectives: Histologic classification along with clinical stage predominantly drive management of patients with endometrial cancer. However, current clinico-pathologic risk-based stratification has proven suboptimal, inciting efforts to identify additional molecular classifiers, such as L1CAM. This is of particular relevance for the TCGA-defined Nonspecific Molecular Profile (NSMP) and MMR-deficient (MMR-d) groups of tumors, both of which are classified as having an intermediate prognosis. In current practice, L1CAM immunostaining is reserved for NSMP tumors that have been classified as MMR-proficient. The aim of this study is to investigate L1CAM testing in tandem, rather than sequential with that of MMR. *Methods*: A total of 149 MMR-tested endometrial carcinoma cases from 2019–2020 were identified, of which, 45 had also undergone L1CAM immunostaining. Clinical information including grade, stage, and treatment was reviewed. This was correlated with percentage of L1CAM positivity and MMR-status. *Results*: L1CAM positivity was noted in 7/45 (15.6%) cases with 6/45 (13.3%) additional cases demonstrating only focal positivity. MMR deficiency was noted in 24/45 (53.3%) of the cases in which L1CAM was performed. Of the cases that showed L1CAM positivity, 6/7 (85.7%), were found to be MMR-deficient. Within the remaining group in which L1CAM was not performed, 24/104 (23.1%) of cases showed MMR deficiency. *Conclusions*: Current findings suggest that L1CAM positivity is not mutually exclusive when correlating with MMR status. Performing L1CAM immunostaining on all endometrial carcinomas may assist in appropriate treatment for patients with L1CAM positivity, and in particular, in MMR-proficient cases classified within the NSMP category.

Keywords

L1CAM; MMR; Endometrial cancer; Testing algorithm; Molecular classification

1. Introduction

As the most commonly diagnosed gynecologic malignancy in the United States, endometrial cancer primary affects postmenopausal women. However, approximately 14% of endometrial cancers are diagnosed in premenopausal women, of which one-third occur in women under the age of 40 years. Unlike most other cancers, the incidence of endometrial cancer (EC) has continued to slowly increase over the past decade with an associated increase in cancer mortality. The increasing incidence has been attributed to the rise in obesity, aging population, changing hormonal risk factors, and more importantly, rising rates of more aggressive, non-endometrioid histology [1]. Efforts to decrease mortality from endometrial cancer need to focus on improved risk stratification at initial diagnosis, identifying those patients at highest risk for recurrence who would benefit from adjuvant treatment and improving therapeutic options for those who develop recurrent disease.

In early stage EC, risk stratification of patients after surgery using clinico-pathologic features typically drive the need for adjuvant therapy. However, stratifying patients into various risk groups based on these criteria continues to be suboptimal [2]. While most early stage, grade 1 and grade 2 endometrioid endometrial carcinomas are associated with good prognosis, a subset of patients with lower stage tumors will still experience relapse and poor outcome [3, 4]. Conversely, approximately 50% of patients with high grade tumors that are classified as high risk experience no recurrence [2]. Additional classifiers, beyond the currently used clinico-pathologic criteria first described in GOG 99, have evolved over the past two decades with the promise of a more clinically reliable stratification scheme.

Molecular classification of EC was first introduced by The Cancer Genomic Atlas (TCGA) using whole genome sequencing in 2013, with 4 distinct subtypes identified including: (1) POLEmut: DNA polymerase epsilon exonuclease (POLE) domain mutations or ultra-mutated carcinoma; (2) MMRd: microsatellite-instable (MSI) hyper-mutated with deficient mismatch repair (MMR) proteins; (3) Copy-number high: p53 mutant; (4) Copy-number low: Nonspecific Molecular Profile (NSMP). These subtypes have been further studied clinically and found to translate into prognostic outcomes. The NSMP or copy number low subtype is defined by the lack of molecular subtype expression that defines each of the other three groups. Prognostic outcomes are less

clear within this subgroup, arguably due to a large amount of heterogeneity within its population. Further molecular stratification of the NSMP subgroup is necessary in order to better classify this group and help to guide adjuvant treatment recommendations. This knowledge has led to further research efforts on alternative molecular classifiers than those developed by the TCGA [5].

One such classifier is L1CAM, which has already been established as an important molecular classifier in NSMP EC because of its ability to identify a subset of tumors within this group who have unfavorable outcomes [3, 6]. Identification of L1CAM in endometrioid endometrial carcinoma may be related to a subtle non-endometrioid (serous, clear-cell differentiation) component, an unfavorable epithelial/mesenchymal transition, or hidden aggressive neuroendocrine elements [3], all of which can translate to poorer clinical outcomes. This suggests that L1CAM should be tested in NSMP tumors which are, by definition, MMR proficient and p53-wild type, and have an intermediate prognosis. This is similar to the MMR-deficient tumors, which are the other intermediate prognosis subgroup classified by TCGA. The testing algorithm proposed by this classification, as well as the suggested Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) algorithm, which was later introduced by Talhouk *et al.* and others [7–9] as a more practical algorithm, uses a sequential molecular classifier testing approach. Under the current proposed testing algorithms, L1CAM immunostaining is reserved for NSMP tumors as a subsequent step following the initial MMR IHC screening. Therefore, L1CAM testing will theoretically only be applicable to tumors that have been classified as MMR-proficient. The aim of this study is to investigate L1CAM testing in tandem with, rather than sequential to, MMR in order to assess whether MMR-d tumors may need further molecular classification for risk stratification.

2. Material and methods

2.1 Patient and tissue selection

After obtaining Institutional Review Board approval, a retrospective review of our electronic pathology database was performed, and 149 patients who underwent hysterectomy with a pathologic diagnosis of endometrial cancer between 2019–2020 were identified. MMR immunohistochemistry had already been established as a reflex test for all patients in our institution in 2019. Therefore, all cases were subjected to MMR immunohistochemistry screening as part of their clinical diagnostic workup. Forty-five cases of endometrioid carcinoma with representative tumor tissue remaining in paraffin blocks were identified for additional L1CAM testing. L1CAM immunohistochemistry was performed on the same tissue block that was used for MMR immunohistochemistry to avoid sampling and possible tumor heterogeneity bias. L1CAM testing was conducted as part of our institutional validation of further molecular classification efforts of endometrial cancer. Patients' age, clinical stage and adjuvant therapy were obtained from the patient charts. The patients were categorized according to histologic subtype and the International Federation of Gynecology and Obstetrics (FIGO) grading system.

2.2 Immunohistochemical staining and expression evaluation

The formalin fixed paraffin-embedded (FFPE) tissue blocks were collected from the archives of the department of anatomic pathology and cut into 4 μm sections which where mounted on Super frost slides. The sections obtained were subjected to immunohistochemistry on a Ventana BenchMark Ultra immunostainer (Ventana Medical Systems). Primary antibodies used: anti-PMS2 (clone A16-4), anti-MLH1 (clone M1), anti-MLH6 (clone SP93) and anti-MSH2 (clone# G219-1129) all from Ventana Medical systems (Tuscan, AZ) and anti-L1CAM (clone 14.10, Biolegend, San Diego, CA). All antibodies used were validated for clinical use on the analyzer and staining was performed following the manufacturer's protocols. Briefly, antigen retrieval was done using CC1 (Ventana) for 32 min (L1CAM), 40 min (MSH2), 64 min (MSH6 and MLH1) and 90 min for PMS2. The antibody incubation times were: 8 min for MSH6, 12 min for MSH2 and 32 min for the remaining antibodies. Antibody amplification was applied for PMS2 using the Ventana Amplification Kit (Ventana Medical Systems). Visualization was achieved using 3′3-diaminobenzidine tetrahydrochloride substrate (DAB) and hematoxylin counterstaining.

L1CAM staining extent was scored as positive ($\geq$10% of cells) vs. negative ($<$10% of cells). This cut-off has previously been reported to result in the strongest model and was confirmed by Bosse *et al.* 2014 [10]. Scoring was performed by a single gynecologic pathologist, who was blinded for clinical outcome and MMR status. The patients' samples were identified as MMR proficient when all the four proteins from MMR panel were expressed.

All cases in this study and the imunohistochemical interpretations were rendered by two board certified pathologists with focused practice on gynecologic pathology for more than 15 years (MEK and MAK). They used the current diagnostic criteria of endometrial cancer diagnosis adopted in their standard daily practice.

3. Results

149 patients who underwent hysterectomy with a pathologic diagnosis of endometrial cancer between 2019–2020 were identified. MMR and L1CAM immunohistochemistry were performed on a total of 45 cases with a pathologic diagnosis of endometrioid endometrial cancer. These patients ranged in age from 28 to 98 years old (median age 63 years). Of the 45 cases, 28 (62.2%) were FIGO grade 1, 7 (15.6%) were FIGO grade 2, and 10 (22.2%) were FIGO grade 3. Four patients had positive lymph nodes and were classified as FIGO stage I.

L1CAM was positive ($>$10% of cells) in 7 of 45 cases (15.6%). Immuno-positive cells typically clustered in defined

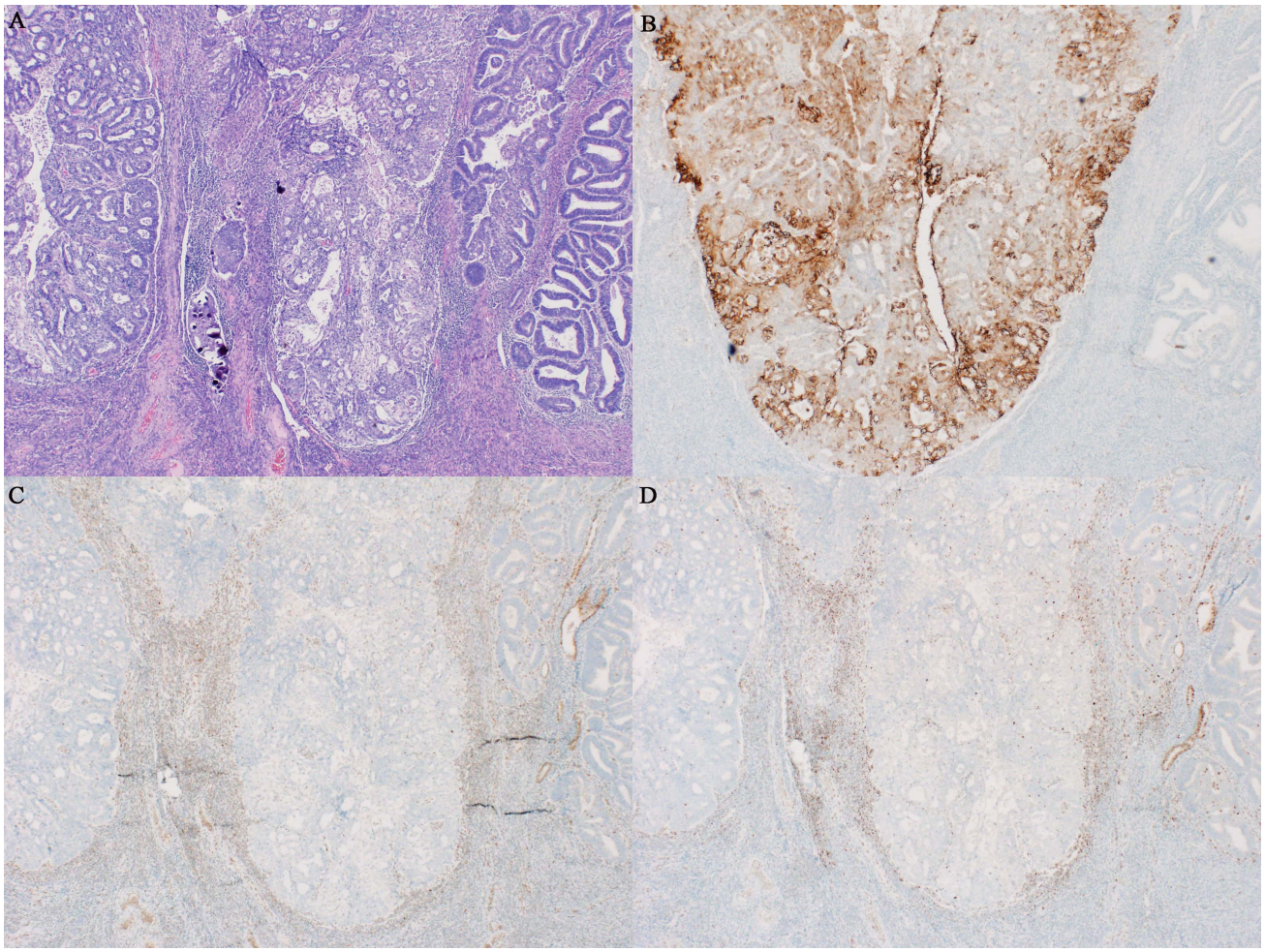

Fig. 1. L1CAM-positive, MMR-d adenocarcinoma. (A) Endometrioid adenocarcinoma with groups of cribriform malignant endometrial glands with no myometrial invasion (H&E × 40). (B) L1CAM-positive immunostaining shown in a cluster of malignant cells representing >10% of the tumor on the section. Negative malignant cells are shown on the right side of the picture (L1CAM × 40). (C) Same cluster of cells with loss of MLH-1 expression. Internal control of non malignant cells is positive (MLH-1 × 40). (D) Same cluster of cells with concomitant loss of PMS-2 expression. Internal control of non malignant cells is positive (PMS-2 × 40).

areas on the tissue section, reflecting the presence of a distinct positive aggressive subclone of malignant cells (Fig. 1). Clusters of positive cells filled the majority of one low-power field of the tumor and could be detected quite easily in the endomyometrial section of the hysterectomy specimen while scanning at a low magnification microscopy. The consistency of this observation between cases highlights the focality of the L1CAM-positive subclone in these tumors and, therefore, the potential for a false negative result in a preoperative biopsy. Cases with positive immunostaining in tumor cells but in <10% of tumor cells were designated as having "focal staining" (seen in 6 of 45 cases) and these cells also clustered but in smaller groups (Fig. 2). Cases with this pattern of staining were considered negative.

Of the seven L1CAM-positive cases, 4/7 (57.1%) were identified as FIGO stage IA. Of the remaining three cases, two were staged as II and one as IIIA. Three of seven cases (42.9%) were classified as FIGO grade 1. Of the remaining four, one was classified as FIGO grade 2 and three were classified as FIGO grade 3 (Table 1).

When comparing L1CAM to MMR findings, 6/7 (85.7%) of L1CAM-positive cases were found in the MMR-deficient group. Conversely, the majority of the cases that were MMR-proficient were found to be L1CAM-negative or focally-positive (18/21, 85.7%) (Table 2). Interestingly, 4 of the 6 (66.7%) L1CAM-focally positive cases were in the MMR-deficient group.

Patient treatment data was compared with IHC analysis. A total of nine (20%) patients received adjuvant treatment with radiation and/or chemotherapy. Incidentally, 2/9 (22.2%) were L1CAM-positive and 7/9 (77.8%) were L1CAM-negative. Four of nine cases (44.4%) showed MLH1 and PMS2 expression loss. Of these deficient cases, two received both chemotherapy and radiation whereas the other two did not get any adjuvant treatment. Four of the nine MMR-deficient cases (44.4%) were FIGO grade 1, two

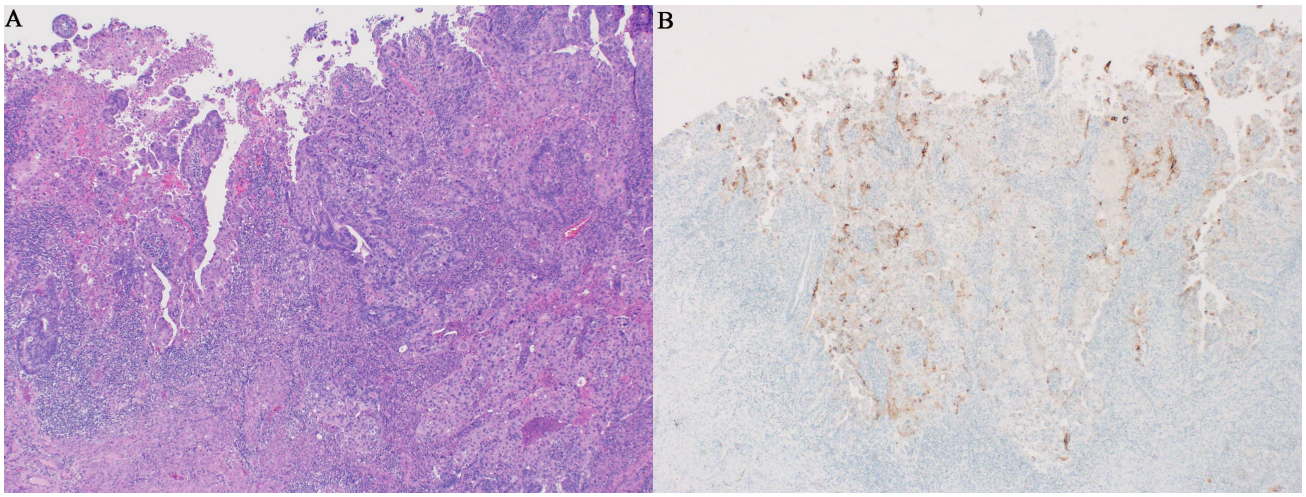

Fig. 2. L1CAM showing focal staining, considered as negative. (A) Endometrioid adenocarcinoma with superficial myometrial invasion on the right side of the photomicrograph (H&E × 40). (B) Only focal, scattered malignant cells are positive for L1CAM representing <10% of malignant cells in the section; the case is interpreted as negative (L1CAM × 40).

Table 1. Immunohistochemical results on 45 cases of endometrioid adenocarcinoma.

Tumor grade	Number (%)	MMR IHC		L1CAM IHC		
		Proficient	Deficient	Negative	Focal	Positive
FIGO 1	28 (62.2%)	18 (64.3%)	10 (35.7%)	24 (85.7%)	1 (3.6%)	3 (10.7%)
FIGO 2	7 (15.6%)	1 (14.3%)	6 (85.7%)	3 (42.9%)	3 (42.9%)	1 (14.2%)
FIGO 3	10 (22.2%)	2 (20%)	8 (80%)	5 (50%)	2 (20%)	3 (30%)
Total	45	21 (46.7%)	24 (53.3%)	32 (71.1%)	6 (13.3%)	7 (15.6%)

Table 2. Results of L1CAM testing correlated with the MMR status.

MMR	L1CAM		Total
	Positive (≥10%)	Focal or Negative	
Proficient	1	20	21
Deficient	6	18	24
Total	7	38	45

Table 3. MMR IHC results of cases with no L1CAM testing.

Tumor grade	Number (%)	MMR IHC	
		Proficient	Deficient
FIGO 1	76 (62.2%)	59 (77.6%)	17 (22.4%)
FIGO 2	14 (15.6%)	9 (64.3%)	5 (35.7%)
FIGO 3	11 (22.2%)	9 (81.8%)	2 (18.2%)
Serous	3 (22.2%)	3 (100%)	0 (0%)
Total	104	80 (76.9%)	24 (23.1%)

(22.2%) were FIGO grade 2, and three (33.3%) were FIGO grade 3. Most of the nine cases (8/9, 88.9%) were staged as IA or IB and only one was staged as IIIA.

104 of the 149 cases that were identified were tested by MMR IHC but L1CAM testing was not performed. Twelve of these patients had positive lymph node metastases. A great majority of these cases were classified as stage pT1a (73/104, 70.2%) and as grade FIGO grade 1 (76/104, 73.1%). A total of 13 cases were classified as pT1b and six as pT2. A total of 14/104 (13.4%) cases were classified as FIGO grade 2 and 11/104 (10.6%) as FIGO grade 3. 3/104 (2.8%) were classified as serous carcinomas. Of the 104 cases, 24 (23.1%) were found to be MMR-deficient and the remaining 80 (76.9%) were MMR-proficient. When broken into tumor grade categories, 17/76 (22.4%), 5/14 (35.7%), and 2/11 (18.2%) of the FIGO grade 1, FIGO grade 2, and FIGO grade 3, respectively, were MMR-deficient. All three serous carcinoma cases were found to be MMR-proficient (Table 3).

4. Discussion

L1CAM is believed to promote aggressive tumor behavior by driving cell proliferation, migration and metastasis [3]. Studies have proven L1CAM positivity to portend a poor prognosis in endometrial cancer (EC) [3], though it has yet to be correlated with MMR findings. It is currently unknown whether L1CAM and MMR status should be tested by immunohistochemistry sequentially or concomitantly.

MMR testing is feasible and routinely performed at a large scale in clinical practice for EC. Subsequent or concomitant molecular testing is not currently routine, but may be imperative to determining risk stratification of early stage, low grade EC patients. The Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) algorithm has been proposed as a clinically feasible and sequential approach to tumor testing in EC [7]. This algorithm does use POLE mutation testing as one of the molecular classifiers. One possible

limitation with this approach is the fact that the identification of POLE mutations requires sequencing, which is both costly and time consuming, possibly resulting in lower use of this algorithm. In addition, genetic sequencing steps are difficult to integrate within the workflow of most surgical pathology laboratories, especially in the community setting, which would argue for a more centralized testing approach in larger academic or commercial laboratories [11]. From our experience in clinical practice, POLE mutation sequencing is not routinely performed for these reasons and improper classification will likely lead to over or under treatment of patients [12]. Another possible limitation of this algorithm is that MMR deficient (MMR-d) patients do not undergo any further molecular testing and it remains to be seen whether MMR-d tumors can be further risk stratified by additional molecular classifiers.

L1CAM is strong prognostic marker that can help to further classify NSMP EC because of its ability to identify a subset of tumors with unfavorable outcomes [3, 6]. It has been hypothesized that L1CAM might be able to detect a subtle non-endometrioid tumor component, unfavorably influence epithelial/mesenchymal transition, or identify hidden aggressive neuroendocrine elements [3]. Our current study suggests that concomitant testing of EC tumors for MMR and L1CAM may be of clinical benefit to ensure that patients with a higher risk tumor profile are not missed with MMR testing alone. In our study, six of the seven L1CAM-positive tumors were detected in the MMR-deficient group which would suggest that their L1CAM status would not have been revealed had these suggested algorithms been applied. Even more, 4 of the 6 L1CAM-focally positive tumors were detected among the MMR-deficient subset. It is important to acknowledge, however, the valid argument that the clinical application of L1CAM focal positivity is questionable as we should limit our positive result interpretation to those cases with $\geq$10% positivity [3]. Additionally, our study shows the value of L1CAM testing in an endomyometrial tissue section from the hysterectomy specimen, as opposed to performing the immunohistochemical test on a preoperative biopsy sample of the endometrium. Scanning at a low magnification allowed the visualization of the clustered cells of the L1CAM positive subclone on the section, which would most likely amount to $\geq$10% of malignant cells. Our results, therefore, emphasize the value of identifying L1CAM-positive tumors, even among MMR-deficient cases which can be achieved by the concomitant testing of the two classifiers at the outset of the case workup, rather than the sequential application of these IHC tests.

It is of note that Stelloo *et al.* [13] observed a lower percentage of L1CAM-positive cases among the MMR-deficient group and this could be merely by chance. We used the same L1CAM clone in this study but was from a different manufacturer. Unlike in the study of Stello *et al.* [13] all of our patients were of the endometrioid cell type. However, further investigation into the appropriate percentage of L1CAM-positive patients in this cohort is needed as this current work is meant to serve a proof of concept.

As care of patients becomes more personalized, tumor categorization is becoming progressively reliant on molecular classification as opposed to the traditional histomorphology [12]. While current practice is predicated on clinicopathologic features, we strive for identifying practical approaches to molecular classifications that can be consistently and reliably applied in the daily practice of community as well as academic pathology practice. While in theory most low grade and low stage carcinomas are thought to show a favorable prognosis, our study as well as those of others [3] showed that L1CAM positivity is also seen in low grade, early-stage EC. More than 10% of FIGO grade 1 EC and 57.1% of stage IA tumors in our series were L1CAM-positive. These observations strongly advocate for performing L1CAM staining in EC, especially those of low grade/stage, irrespective of or their MMR status. Performing L1CAM immunostaining may be particularly relevant in the MMR-proficient and p53-wild type, or NSMP, tumors. However, results of this study also highlight the risk of missing the L1CAM-positive tumors among MMR-deficient cases. Further research on prognostic outcomes is required to determine if L1CAM status in MMR-d tumors is relevant.

MMR-deficient tumors comprise approximately 36% of endometrial carcinomas and MLH1 and PMS2 loss was noted in 29.8% of these cases [14–16]. Cases in our study showed a comparative rate of 23.1% (24/104) of MMR deficiency in the 104 patients for whom MMR immunohistochemistry was reflexively performed. MMR-proficiency in the control group is more or less consistent with that found in other studies. Our cohort of 45 cases in which L1CAM immunostaining was performed show that 24/45 (53.3%) cases were MMR-deficient.

Limitations include a relatively small cohort size, though the primary objective of this study is a proof of principle at this time making sample size less important. Additionally, the study is retrospective in nature and has inherent limitations secondary to this.

Initiation of the discussion around the concept of sequential testing of EC is emphasized with this study and makes an argument for moving L1CAM testing up in the algorithmic approach, placing it in tandem with MMR testing. Findings in our study showed that L1CAM positivity is not mutually exclusive when correlating with MMR status. Performing L1CAM immunostaining on all endometrial carcinomas may assist in risk stratification and appropriate treatment for patients with L1CAM positivity, particularly in the MMR-d and NSMP subgroups.

5. Conclusions

In conclusion, integration of molecular risk factors with clinicopathologic factors in early-stage endometrial carcinoma leads to improved risk stratification with potential therapeutic utility. This study presents a proof of concept

supporting the concurrent testing of L1CAM and MMR proteins as a valuable tool in risk stratification of endometrial cancer.

Author contributions

JC wrote the manuscript and analyzed the data. MH collected the data, examined patients charts, and reviewed the manuscript. MF performed the technical work, wrote the methods, and reviewed the manuscript. JM, SAM, and BW contributed the clinical part of the study, reviewed and finalized the manuscript. MEK diagnosed the cases, supervised the study and reviewed the manuscript. MAK the senior author who designed the study, supervised and approved the manuscript and is the corresponding author.

Ethics approval and consent to participate

The Institutional Review Board of the University of Minnesota granted the researchers an exemption and approved this study on August 6, 2020 under the number STUDY00010497.

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Conflict of interest

The authors declare no conflict of interest.

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