

Immunohistochemical mismatch repair deficiency versus PCR microsatellite instability: a tale of two methodologies in endometrial carcinomas

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Summary

Endometrial carcinomas are common gynecological malignancies. Microsatellite instability and mismatch repair deficiency may be detected in endometrial carcinomas and tumours associated with Lynch syndrome. We aimed to compare results obtained using immunohistochemical mismatch repair (MMR) antibodies and polymerase chain reaction (PCR) for microsatellite instability (MSI) on endometrioid endometrial carcinomas (EEC) at a South African state hospital. Once ethical clearance was obtained, 145 cases of EEC were retrieved. These cases were subjected to immunohistochemistry (IHC) for MLH1, PMS2, MSH6 and MSH2 antibodies; and multiplex MSI PCR for the markers BAT-25, BAT-26, NR-27, NR-24, and NR-21. Cases demonstrating MMR and MSI discordance and cases showing loss of MLH1 staining then underwent MLH1 promoter methylation testing. Mismatch repair deficiency was noted in 28.28% of 145 cases, whilst 37.1% showed MSI by PCR. The overall accuracy was 69.29%. There were 37 cases showing loss of MLH1 staining and MMR/MSI discordance was detected in 25 cases. These cases underwent hypermethylation assessment which was identified in 72.13% of cases. The current study shows that 25 (17.24%) out of 145 cases would not have had abnormalities identified if PCR MSI had not been performed and would not have been flagged as having a possible germline mutation. Most (68%) of these 25 cases were hypermethylated. We therefore recommend that endometrial carcinomas undergo both screening tests in South Africa for patients under the age of 70 years. Tissue specimens may be tested for MSH6 and PMS2 immunohistochemical stains in addition to PCR MSI testing.

Key words: Endometrial carcinomas; Immunohistochemical mismatch repair deficiency; PCR microsatellite instability.

Introduction

In western countries, the most common malignancy of the female genital tract is endometrial carcinoma, the incidence of which is increasing annually [1, 2]. The most recently published statistics from the South African National Cancer Registry in 2016 recorded an incidence of 3.42% of uterine malignancies amongst all registered tumours in females [3].

Endometrioid endometrial carcinomas often demonstrate microsatellite instability which is less common in other histological subtypes [4-10]. Microsatellites are DNA base pair sequences that are repeated multiple times and are highly susceptible to changes in mismatch repair [4, 11, 12]. A DNA mismatch repair system (MMR) detects any replication error that may occur on microsatellite areas and will correct these [13-17]. MMR genes include *MLH1*, *MSH2*, *PMS2*, *MSH3*, *MSH6* and others [16, 17]. If there is functional inactivity of any of the four proteins (*MLH1*, *PMS2*, *MHS2* and *MSH6*), mismatch repair will not occur [18].

Lynch syndrome (LS), is an autosomal dominant syndrome caused by mutations in the DNA MMR system in

which affected individuals have an increased susceptibility to development of endometrial carcinomas as well as other tumours [15-21]. Immunohistochemical testing for *MLH1*, *MSH2*, *PMS2* and *MSH6* proteins on formalin fixed paraffin embedded (FFPE) tissue has been suggested as the screening test of choice to identify patients with LS as immunohistochemistry is available in most anatomical pathology laboratories [22, 23].

Microsatellite instability (MSI) may be detected by polymerase chain reaction (PCR) assays which require assessment of an individual's tumour and non-tumour tissue. Several markers may be used in MSI testing. There may be lengthening or shortening of DNA sequences due to insertion or deletions of repeated nucleotides in tumours when compared to non-neoplastic tissue [24]. Microsatellite instability is assessed on the sizes of neoplastic PCR products in comparison to the patient's normal tissue [25]. It has been suggested that MSI testing has an advantage over IHC testing as molecular pathology personnel may interpret PCR results whereas pathologists are required for IHC evaluation [26]. Furthermore, use of multiplex or penta-

plex PCR may allow for increased through-put as multiple markers may be amplified in a single reaction whilst simultaneously decreasing costs of labour and consumables [27]. MSI PCR assesses functionality of MMR genes. Therefore, MSI PCR may detect cases which are regarded as mismatch repair proficient on an immunohistochemical protein level but are in fact due to missense mutations in MMR genes. Thus, such a mutation would not be detected immunohistochemically but can be identified by PCR [28].

McConechy *et al.* have demonstrated a high level of concordance (93%) between IHC MMR and MSI by PCR on endometrial carcinomas [29]. To the authors' best knowledge, such assessments have not been undertaken on endometrial carcinomas in South Africa.

Materials and Methods

According to international data, approximately 6% of females develop endometrial carcinomas, and 15-30% of these patients may have microsatellite unstable tumours [30]. In order to observe a significant difference ($p < 0.05$) at 80% power between the two groups, a sample size of 145 was required. Once ethical clearance (clearance certificate number M151051) was granted by our university's Human Research Ethics Committee (Medical), 145 cases of endometrioid endometrial carcinoma were retrieved from departmental archives for the period 2009-2015. This study began in 2015 and as such, cases prior to this time frame were retrieved. The cases were reviewed by an experienced anatomical pathologist who confirmed endometrioid morphology in all endometrial carcinomas.

Immunohistochemistry

Immunohistochemistry was performed on 4 μ m deparaffinised sections using the MMR antibodies; MLH1 (Novocastra, UK, Clone ES05, 1 : 50), PMS2 (Novocastra, UK, Clone MOR4G, 1 : 50), MSH2 (Novocastra, UK, Clone 25D12, 1 : 50) and MSH6 (Novocastra, UK, PU29, 1 : 50) according to departmental standard operating procedure and manufacturer instructions. Appropriate positive and negative control tissue sections were used. The tissue sections were assessed in a binary manner such that there was retention of tumour nuclei staining (regardless of intensity) or loss of staining of tumour cells, whilst in the presence of internal positive control staining of endothelial cells, lymphocytes and stromal cells.

MSI PCR testing

The methods for multiplex PCR and primers used for amplification of microsatellite sequences in this study were those previously utilised by Haghghi *et al.* and comprised BAT-25, BAT-26, NR-21, NR-24 and NR-27 [31]. Tissue sections containing tumour and non-tumour patient DNA were cut from each paraffin-embedded block. Following PCR, the size of alleles from the tumour were compared to patient non-tumour DNA in the tissue sections. Cases demonstrating a difference in size of alleles in one out of the five markers were deemed MSI-Low (MSI-L) whilst

cases showing no difference in size were interpreted as microsatellite stable. Cases illustrating differences in allele sizes in two or more of the five markers were considered MSI-High (MSI-H) [13-15].

MLH-1 hypermethylation using MassARRAY EpiTYPER

Quantitative *MLH1* Promoter hypermethylation analysis was performed using MassARRAY EpiTyper analysis, by Agena Bioscience at Inqaba Biotec. EpiTYPER provides reliable results and has several components including data analysis and reporting software for graphic representation of quantitative methylation at each CpG site [32].

Based on a study by Pérez-Carbonell, the target sequence of the *MLH1* promoter region, (-248 to -178) was used [33]. The forward primers were: AGGAA-GAGCGGATAGCGATTT and the reverse primers were: TCTTCGTCCCTCCCTAAAACG [33]. These primers allowed for the best possible coverage. A 187 base pair product size was generated and 11 CpG islands could be assessed. Eight CpG sites were evaluated. However, due to lower mass cleavage products, 3 CpG target sites could not be examined. The inability of EpiTYPER to evaluate samples with a lower mass cleavage product is an established shortcoming of this system [34]. Patient DNA extracted from paraffin embedded wax blocks underwent bisulphite conversion, PCR, transcription and cleavage, as well as detection of cleaved products by MassARRAY according to the manufacturer's instructions and Ehrich *et al.* [32].

Statistical analyses were undertaken utilising STATA version 15 (StataCorp LP, College Station TX). Sensitivity and specificity analyses assessed levels of sensitivity of each of the procedures in comparison to tests regarded as the gold standard [35]. Cohen's Kappa evaluated concordance/agreement between categorical (qualitative) variables (which were the two test methods, namely IHC and PCR for microsatellite instability). Pearson's correlation was used to measure the strength of the relationship (association) between two continuous and normally distributed variables (IHC staining and methylation status; in addition to PCR assessment and methylation status).

Table 1. — Mismatch repair deficiencies per immunohistochemical marker.

| IHCs | MMR Deficient n = 145 (%) |
|----------------------------|---------------------------|
| MLH1 only | 16 (11.03) |
| MLH1 and PMS2 | 20 (13.79) |
| MSH6 only | 2 (1.38) |
| MSH6 and MSH2 | 2 (1.38) |
| MSH6, MLH1 and PMS2 | 1 (0.69) |
| Total | 41 (28.28) |

Results

Immunohistochemistry

Out of 145 cases, a total of 41 (28.28%) samples showed loss of nuclear staining and were thus considered mismatch repair deficient. The results are depicted in Table 1 and Figure 1.

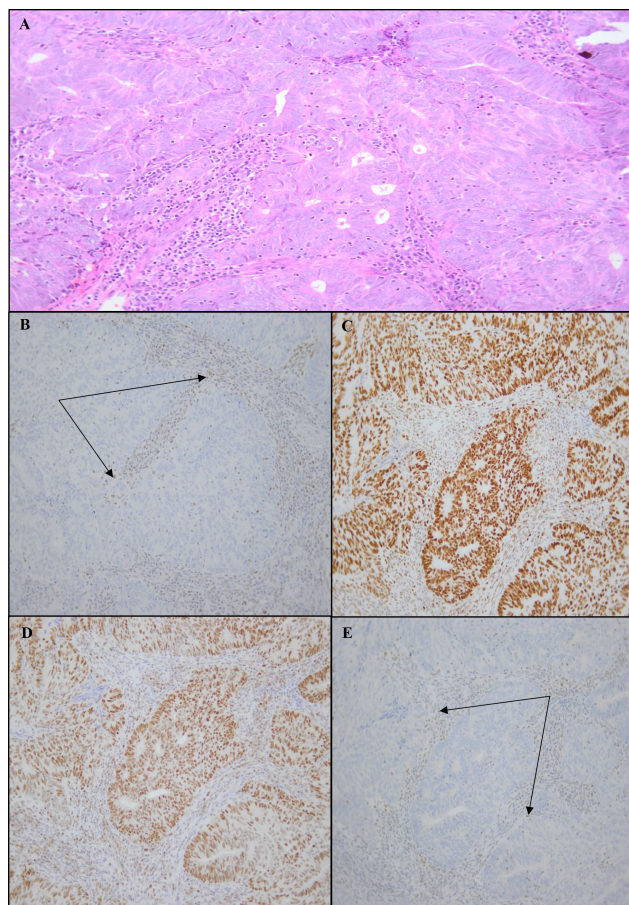


Figure 1. — Microscopic images of the tumour and immunohistochemical stains. (A) Haematoxylin and Eosin stained section of an endometrioid endometrial carcinoma. (B) MLH1 immunohistochemical stain showing retained staining of lymphocytes (arrows) but loss of staining of tumour nuclei. (C) MSH2 and (D) MSH6 immunohistochemical stains show retained staining of tumour nuclei. (E) PMS2 immunohistochemical stain showing loss of staining in tumour nuclei whilst lymphocytes and stromal cells are positive (arrows). All images at 200 \times magnification.

MSI PCR

Microsatellite instability was detected in 46 (37.1%) cases by PCR. Of these 46 cases, 24.1% were microsatellite low and 7.6% were microsatellite high. Ninety-four (64.8%) cases were microsatellite stable. There were 34 (23.45%) cases that had 1 mutation identified out of the five tested markers. Twelve (8.28%) cases had 2 or more mutations out of the 5 markers examined and were thus in-

terpreted as microsatellite high. Despite PCR having been undertaken on three occasions, there were 5 cases (3.5%) that yielded no PCR reaction.

Using both IHC MMR and PCR MSI identified abnormalities in 45.51% of cases; whilst 54.48% of cases showed no abnormality by either test.

MMR IHC versus MSI PCR

Forty-one (28.28%) cases showed deficient immunohistochemical staining, whereas 46 (31.72%) out of 145 cases were microsatellite unstable by PCR. For comparative purposes, the 5 cases in which no result was obtained by PCR were excluded from further assessment.

Twenty-one out of 140 cases demonstrated abnormalities by both PCR and IHC. Twenty-five cases showed retained IHC staining but were unstable by PCR. Seventy-six cases were stable by PCR and were MMR proficient. The total number of cases that were MMR deficient, was 39. The total number of cases that were microsatellite unstable by PCR, was 46. The 25 discordant IHC/PCR cases subsequently underwent methylation assessment by EpiTYPER which showed methylation in 68% of cases (Figure 2). The sensitivity of IHC was 45.65%; 95% CI (37.40-53.90), the specificity was 80.85%, 95% CI (74.33-87.37). The positive predictive value (PPV) was 53.85%, 95% CI (45.59-62.11) and the negative predictive value (NPV) was 75.25%, 95% CI (68.10-82.40). The overall accuracy was 69.29%.

The 37 cases that were deficient for MLH1 and/or PMS2 by immunohistochemistry, in addition to the 25 discordant IHC/PCR results underwent methylation analysis of the MLH1 promoter region. Thus, 62 cases underwent EpiTYPER analysis with only one case having had insufficient DNA for analysis and was thus excluded for comparative purposes. A value of $\geq 10\%$ was regarded as hypermethylated [36]. Table 2 shows that DNA from 27.87% of cases was not methylated whereas DNA from 72.13% of cases was hypermethylated.

Seventeen (68%) out of 25 discordant MMR/MSI cases showed hypermethylation. From the residual 8 MSI/MMR discordant cases, 7 were unmethylated. One case had insufficient quantities of tissue from the paraffin embedded block and could thus not undergo methylation analysis.

Table 3 shows that 70.59% of 61 cases had no methylation or loss of IHC staining; whilst nearly 30% of cases that had intact MMR IHC staining were methylated. There were 2 cases which yielded no result by PCR and as such, for comparative purposes with methylation analysis, were excluded, thus bringing the total to 59 cases. Table 4 shows that 12.50% of cases which had no PCR mutation were not methylated, whereas approximately a third of cases that were methylated were microsatellite stable. Of the 16 cases that were not methylated, 87.50% showed MSI by PCR, whereas two-thirds of methylated cases had microsatellite instability.

Table 5 shows that of the unmethylated cases, 12.50% were microsatellite stable; whilst the majority (81.25%)

Table 2. — EpiTYPER testing on sixty-one cases.

| EiTYPER | Frequency (%) | Total | Std error | 95% Conf Interval | p-value |
|--------------|---------------|-------|-----------|-------------------|---------|
| Unmethylated | 17 (27.87) | 27.87 | 2.265088 | 61.90411 71.50765 | |
| Methylated | 44 (72.13) | 72.13 | 1.356642 | 62.62771 68.09957 | 0.6964* |
| Total | 61 (100) | 100 | 1.157041 | 63.42328 68.05213 | |

Table 3. — Methylation by EpiTYPER compared to IHC staining.

| EpiTYPER METHYLATION | | | | |
|-----------------------|-------------------|----------------|-------------|-------------|
| | | No Methylation | Methylation | Total |
| Immunohisto-chemistry | Retained staining | 12 (70.59%) | 13 (29.55%) | 25 (40.98%) |
| | Loss of staining | 5 (29.41) | 31 (70.45%) | 36 (59.02) |
| | Total | 17 (100%) | 44 (100%) | 61 (100%) |

Pearson chi2 (1) = 8.5401 Pr = 0.003.

Methylation of discordant MMR/MSI cases

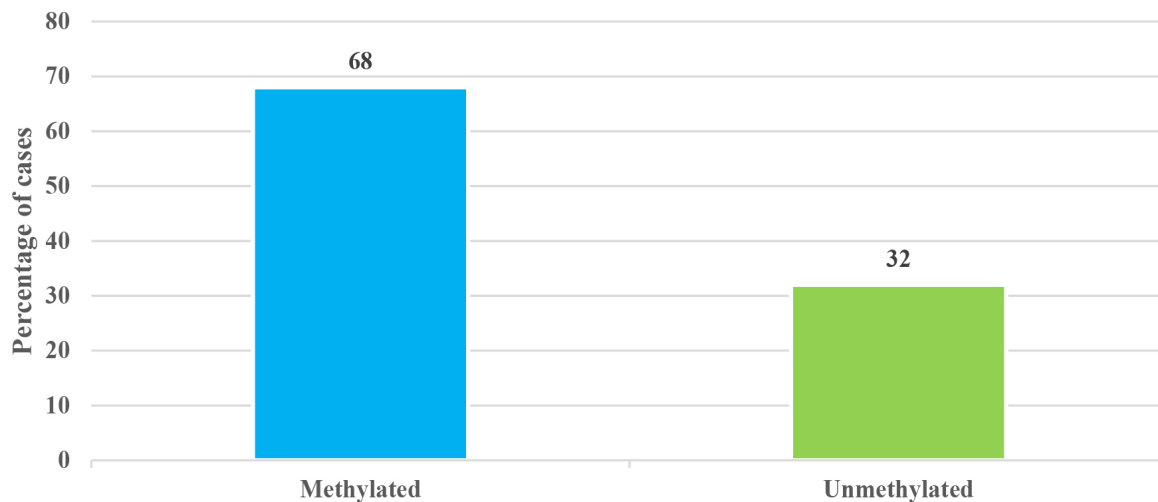


Figure 2. — Methylation of cases that demonstrated MMR/MSI discordance.

Table 4. — Concordance between EpiTYPER and PCR testing.

| EpiTYPER METHYLATION | | | | |
|----------------------|-------------|----------------|-------------|-------------|
| | | No Methylation | Methylation | Total |
| PCR | No Mutation | 2 (12.50%) | 14 (32.56%) | 16 (27.12%) |
| | Mutation | 14 (87.50%) | 29 (67.44%) | 43 (72.88%) |
| | Total | 16 (100%) | 43 (100%) | 59 (100%) |

were MSI-Low and only 1 case was MSI-High. Approximately a third of methylated cases were microsatellite stable. Nearly 47% of methylated cases were MSI-Low whilst one-fifth of methylated cases were MSI-High. The p-value is tending toward statistical significance ($p = 0.057$).

Discussion

Studies by McConechy *et al.* have demonstrated a concordance level of over 93% using MMR IHC and MSI PCR on endometrial carcinomas [29]. In the present study however, there was a concordance level of only 69.29% between the two test methods. There were 25 cases that were microsatellite unstable by PCR but were MMR proficient using IHC, which are interpreted as false negatives when PCR is regarded as the gold standard [37]. Most 17/25 (68%) of the IHC/PCR discordant results were due to hypermethylation. This highlights the loss of staining for MLH1/PMS2 with microsatellite stability on PCR. The mutations identified in the 8/25 residual (7 unmethylated and 1 insufficient DNA) cases may have been identified by PCR due to the ability of the mononucleotide markers to detect small base pair changes. Had MMR IHC testing been the only method used, these cases would have been missed. Cho *et al.* have noted that approximately 50% of

Table 5. — Concordance between EpiTYPER methylation and PCR assessment.

| EpiTYPER METHYLATION | | | | |
|----------------------|-----------------------|----------------|-------------|-------------|
| | | No Methylation | Methylation | Total |
| PCR | Microsatellite stable | 2 (12.50%) | 14 (32.56%) | 16 (27.12%) |
| | MSI-Low | 13 (81.25%) | 20 (46.51%) | 33 (16.95%) |
| | MSI-High | 1 (6.25%) | 9 (20.93%) | 10 (16.95%) |
| | Total | 16 (100%) | 43 (100%) | 59 (100%) |

Pearson $\chi^2(2) = 5.7286$ Pr = 0.057.

patients with MMR-deficiencies do not have germline mutations [22]. This may be attributed to a number of factors such as false positive MMR IHC stains, somatic inactivation of both MMR genes, or the existence of unidentified mismatch repair gene mutations [22]. Other factors implicated in the discrepant results in the present study include, possible germline mutations of the MMR genes, somatic mutations in *MLH1*, use of archived material which had been fixed with non-standard fixation methods, antigen degradation in tissue sections, the possibility of non-functional MMR genes and the possible occurrence of tumour heterogeneity and subclones of tumours which may exhibit loss of immunohistochemical staining in contrast to the surrounding tumour [22, 29, 38-40]. Furthermore, small changes in base pairs may have been identified by PCR [1, 29, 38]. Moreover, it is well-recognised that patients who are carriers of MSH6 mutations are less likely to demonstrate microsatellite instability by PCR; and these cases may be microsatellite stable or MSI-low [29].

Sari *et al.* have proposed that cases showing a weak pattern of nuclear staining be interpreted as mismatch repair deficient as these tumours may be MSI-high tumours; which could be associated with germline mutations [41]. Technical factors such as the interval from biopsy or excision to placement in formalin, the type and pH of formalin used for tissue fixation, in addition to the duration of tissue fixation may also contribute to false positive immunohistochemical results [38, 42-44]. Non-specific background staining, antigen retrieval method and concentration of antibody used, period of primary antibody incubation period and detection kit used are other factors that contribute to false-positive results [43, 45]. In the present study, the interval from surgical biopsy to formalin fixation is a variable that cannot be accounted for by laboratory personnel and is a confounding factor. Our laboratory provides a service to a large catchment area in the southern Gauteng region and as such, it is not possible to control time taken for specimens to arrive at our department. We currently purchase commercially available formalin, but previously produced in-house 10% buffered neutral formalin which may account for the discrepant cases noted on archived material from 2009. The other technical causes for false-positive staining are unlikely to have caused discrepant results as the concentration of antibody used was the same for each of the individual stains and control tissue sections were simulta-

neously run with test tissue sections, all of which were examined for adequacy of staining. Additional technical factors such as antigen retrieval and the detection kit used were the same for all cases evaluated and were thus standardised in this regard. The antibody clones used have been considered as possible causes of discordant results. The MLH1 (clone ES05) antibody was assessed to perform the best amongst a number of clones in an evaluation of MLH1 by NordiQC [46]. Clones for PMS2 (clone MOR4G), MSH2 (clone 25D12) and MSH6 (clone PU29) have not been assessed to be the best clones for their respective antibodies by NordiQC [47-49]. However, these antibodies have been optimised for diagnostic use in our laboratory and have shown appropriate staining of internal controls such as stromal cells, endothelial cells and lymphocytes in both control and test tissue samples. Whilst isolated MLH1 loss is not a common finding, it has been documented by Hashmi *et al.* [50] In the current study, neither punctate nuclear staining (a documented technical artefact), nor membranous/cytoplasmic staining was detected [51]. The identification of uniform nuclear staining in the current study was interpreted as demonstrating an intact MMR system whilst in the presence of positive staining of stromal cells in areas that allowed for distinction from artefactual weak staining. This serves as an explanation for the identification of retained PMS2 staining despite MLH1 loss. Furthermore, it has been documented that missense mutations may give rise to inactive mutant proteins with preserved antigenicity [23]. However, since mutational assessment was not performed in this study, this phenomenon may not be confirmed. Nevertheless, the present study suggests that there is a slight prospect of PMS2 staining in the absence of MLH1.

A study by de Leeuw *et al.* [52] showed that only some cases which had germline MLH1 mutations demonstrated complete loss of MLH1 staining immunohistochemically [52, 53]. Zigelboim *et al.* [54] have identified a deletion mutation in exon 14-15 of MLH1 that resulted in an epitope-stable carboxyl terminal of MLH1 to be deficient in the region of the carboxyl terminal domain which is necessary for adequate interplay between MLH1 and PMS2 with PMS2 stabilization [55]. There was thus failure of the MLH1-PMS2 heterodimer with resultant deficient PMS2 staining; whereas MLH1 protein was still expressed as the epitope was stable and thus immunoreactive [54]. This identifies a limitation of IHC in the preliminary screening of suspected

Lynch syndrome in individuals who have PMS2 deficient tumours without an identifiable PMS2 mutation [54].

If PCR had not been undertaken on all 145 cases in the present study, microsatellite unstable cases diagnosed by PCR would not have been recognised as these cases demonstrated intact immunohistochemical staining. Kato *et al.* have noted that heterogeneous staining patterns are difficult to interpret and as such there has been dismissal of staining interpretation other than to note the presence or absence of nuclear staining [20]. Such variation in staining patterns may be documented in MLH1 and it is thought that germline mutations in *MLH1* may account for such staining [20, 39, 56]. However, it is not possible to determine if a germline mutation is the cause of variable staining in laboratories where germline mutational analysis is not available. Wong *et al.* have recently stated that focal, weak or heterogeneous staining patterns should be interpreted as equivocal or impaired staining and suggest that immunohistochemistry be repeated on an alternate tissue section if this is available [57]. This allows for possible identification of an individual at risk for Lynch syndrome. Watson *et al.* [55] noted that approximately 10% of patients had a false positive result on IHC, which is similar to that noted in the present study where 18 cases (12.41%) of IHC results were identified when compared to MSI PCR results. As raised by Watson *et al.* [55] in resource constrained settings additional MSI testing by PCR may not be warranted; yet, this could imply missing diagnoses of patients and possibly their family members, who may develop a hereditary tumour syndrome.

Whilst BAT26 is considered the best marker to identify MSI-high tumours, Cicek *et al.* have suggested that there may be somatic deletions of the BAT26 MSI marker in cases where there have been germline MSH2 gene mutations [58]. A tumour may then be incorrectly assigned microsatellite stable if only this marker is selected. It is therefore prudent for a panel of markers to be used in the work-up of MSI [59].

The current findings of hypermethylation of MMR deficient cases mirror those of Bruegl *et al.* [60].

It is possible that the 30% of methylated but mismatch repair proficient cases had missense or truncated mutations which resulted in antigenically identifiable epitopes by IHC but had *MLH1* methylation as suggested by Shia [61]. The MMR deficient cases which had no methylation may be ascribed to possible mutations occurring in *MSH2/MSH6* as well as *MLH1* mutations. These findings are suggestive of possible germline mutations as documented by Wang and colleagues [28].

Over two-thirds of cases were concordant for methylation and MSI; but a third of cases were methylated yet were microsatellite stable by PCR. These results support studies indicating a strong correlation between *MLH1* methylation and MSI [62, 63]. The MSS/hypermethylation discordant results may be due to a single allele producing sufficient *MLH1* or may be ascribed to there being a small percentage

of cells which had abnormal MLH1 protein expression due to hypermethylation [1].

Resnick *et al.* have established that use of IHC as a screening modality followed by directed genetic testing is a cost-effective process to identify patients with Lynch syndrome [64]. PCR identified 5.52% cases over and above IHC testing. Whilst this may not seem like a high percentage, the morbidity and mortality implications for the affected individuals and their families are significant. Garg and Soslow have noted that immunochemistry alone may not detect mutations in the 4 marker panel [65]. Therefore, a combination of IHC and PCR should be considered as screening tools for Lynch syndrome in patients with endometrial carcinoma [53, 65].

Whilst reflex testing of all endometrial carcinomas occurs in some institutions, this does not occur in all centres worldwide. The present study suggests a need to screen possible Lynch syndrome patients in the South African population. Cho *et al.* advocate the use of PMS2 and MSH6 immunohistochemical stains [22]. With respect to the discordant IHC MMR/PCR MSI results and the fact that 5% of patients would not have been identified had they not undergone PCR MSI analysis, we recommend that endometrial carcinomas undergo both screening tests at our institution. However, in an attempt to curtail costs, it is suggested that screening be performed in patients under the age of 70 years and that biopsies from such patients undergo IHC testing for MSH6 and PMS2, as suggested by Cho *et al.* together with PCR MSI testing [22].

Cases demonstrating *MLH1* promoter methylation suggest a sporadic occurrence and do not warrant additional molecular assessment. However, cases suspected of harbouring a germline mutation may be offered genetic counselling with a view to mutational assessment (S1, supplementary table). In the current era of personalised medicine, identification of mismatch repair mutations offers the opportunity for use of an antibody directed against programmed cell death (PD-1) [8, 66]. The Food and Drug Administration (FDA) have approved the use of Pembrolizumab, an anti PD-1 drug, for metastatic or unresectable MMR deficient tumours or microsatellite high malignancies that have progressed regardless of previous therapy and for those with no alternate treatment options [67].

It is envisaged that this study will heighten awareness of the possible occurrence of Lynch syndrome in endometrial carcinomas from patients in Sub-Saharan Africa and the African continent. This may then facilitate identification of index patients with Lynch syndrome, affording them and their family members surveillance for synchronous or metachronous tumours.

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Authors' contributions

RW contributed to the conception, design, drafting of manuscript and critically reviewed the manuscript for relevant intellectual content.

WG contributed to the supervision of the study and reviewed the intellectual content of the write-up.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at <https://ejgo.imrpres.com/EN/10.31083/j.ejgo.2020.06.2186>.

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