BRAF testing in a South African cohort of MLH1 deficient endometrial carcinomas: lessons learnt

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Introduction: Endometrial carcinomas are common female genital tract malignancies. Western countries identified BRAF mutations in very few endometrial carcinomas, whilst an Eastern study documented mutations in one-fifth of endometrial carcinomas. We aimed to assess BRAF mutations in an ethnically mixed South African patient population using immunohistochemistry (IHC), polymerase chain reaction (PCR) and Sanger sequencing in relation to MLH1 methylation.

Methods: A total of 145 endometrioid endometrial carcinoma cases were retrieved from departmental archives and underwent MLH1, MSH2, MSH6 and PMS2 IHC testing. The 37 cases that showed MLH1 loss underwent BRAF IHC, PCR and Sanger sequencing.

Results: Six out of 37 cases demonstrated BRAF mutations: 4 were identified by PCR, whilst IHC and Sanger sequencing identified one mutation each. Three PCR mutations were at the V600E codon, whilst 1 case had a V600D mutation. Agreement between IHC versus overall BRAF mutational status, and sequencing versus overall mutational status, was 86.49% (p-value < 0.005). There was agreement of 94.59% between PCR and overall mutational status with statistically significant, moderate non-random concordance (kappa = 0.77, p = 0.0001).

Discussion: BRAF mutations were identified in 16.21% of cases, which is higher than frequencies noted in Western studies, but less than in an Eastern study. There was no association between MLH1 methylation and BRAF status in endometrial carcinomas from our patient population (kappa = −0.0223, p = 0.6649). Our results in endometrial carcinomas, similar to findings from Western studies, indicate that BRAF mutations are not beneficial in distinguishing which patients have spontaneously occurring tumours from those who may harbour germline mutations and are suspected of having Lynch syndrome.

Keywords: BRAF immunohistochemistry, endometrial carcinomas, PCR, Sanger sequencing

BRAF is a subtype of RAF protein that has serine-threonine kinase activity, and forms part of the RAS/RAF/MAP/ERK pathway that culminates in cell proliferation.1 Activity of RAF is usually under control of RAS. Constitutive activation of the RAS/RAF/MAP/ERK pathway occurs due to mutations in BRAF, resulting in carcinogenesis. Studies have shown that BRAF V600E codon mutations result in ten times the tyrosine kinase activity in contrast to wild-type BRAF.2 Tyrosine kinases are involved in cellular communication and assist in the regulation of cell growth and differentiation of cells. Thus, tyrosine kinase dysregulation culminates in an assortment of disorders, in particular neoplasia.3 The role of BRAF mutations in tumours such as malignant melanomas and colorectal carcinomas is well established and such mutations are identified in up to half of colorectal carcinomas.4 The presence of MLH1 promoter hypermethylation, as well as mutations in BRAF V600E, suggests sporadic occurrence of a colorectal neoplasm.5

Tumours with BRAF mutations are mutually exclusive with KRAS mutations such that either, but generally not both, may be found in a tumour. This implies that the two mutations do not confer an added growth benefit to the tumour.6,7 Exon 15 codon V600E and exon 11 are implicated in BRAF mutations.1 Lynch syndrome is a hereditary tumour predisposition syndrome caused by germline mutations in the mismatch repair system resulting in colonic and endometrial carcinomas amongst other tumours. Patients with colorectal carcinoma suspected of having this syndrome may undergo screening by immunohistochemical mismatch repair, microsatellite instability evaluation by polymerase chain reaction and methylation assessment of the MLH1 promoter region together with BRAF mutational assessment.1,5 Identification of MLH1 promoter methylation and BRAF mutations in colonic carcinomas suggest sporadic carcinogenesis as opposed to a germline-associated malignancy.5 A third of sporadically occurring microsatellite colorectal carcinomas and up to 75% of colorectal carcinomas with mutations in the promoter region of MLH1 have been shown to have BRAF V600E mutations. BRAF has consequently been considered the gene of interest in cases of abnormal mismatch repair.2 The identification of a BRAF mutation allows for use of target treatment, which, in the time of precision medicine, is advantageous for the affected patient.8 In endometrial carcinomas, differing results on the role of BRAF mutations have been documented in studies from the West versus those from the East.1,5 Metcalf and Spurdle5 have reported BRAF mutations in just 4% of endometrial carcinomas whilst Feng et al.1 noted BRAF mutations in 21% of endometrial carcinomas. In light of minimal investigative work-up having been undertaken on endometrial carcinomas in the South African population, it is plausible that our ethnically mixed population may harbour mutations not previously identified or occurring at frequencies not previously recorded. As such, simulating testing undertaken in colorectal carcinomas where
BRAF testing may be undertaken on MLH1 deficient cases, we aimed to investigate the presence of BRAF mutations in a cohort of endometrial carcinomas in a South African population using immunohistochemistry, polymerase chain reaction (PCR) and Sanger sequencing. In addition, hypermethylation of the MLH1 promoter region was assessed.

Methods
After obtaining ethical clearance from our university’s Human Research Ethics Committee (clearance certificate number M151051), 145 cases of endometrioid endometrial carcinoma were retrieved from archives of the Department of Anatomical Pathology at the University of the Witwatersrand. Only cases of endometrioid endometrial carcinoma were included in this study as microsatellite instability is largely confined to this histological endometrial carcinoma subtype. The slides were reviewed by an experienced pathologist and the diagnoses confirmed. All cases underwent immunohistochemical mismatch repair assessment for the four antibodies (MLH1, PMS2, MSH2 and MSH6) as this was one of the aims of a broader PhD study. Cases demonstrating a loss of MLH1 staining in tumour nuclei whilst in the presence of appropriate internal control staining subsequently underwent BRAF testing by immunohistochemistry, PCR and Sanger sequencing. To curtail costs, BRAF assessments were restricted to MLH1-deficient cases. As such, BRAF testing was not performed on all endometrial carcinomas. BRAF testing thus formed one investigation as part of a broader PhD study.

Immunohistochemistry
Immunohistochemistry was performed on 4 μm deparaffinised sections using MLH1 (Novocastra, UK, Clone ES05, 1:50), PMS2 (Novocastra, UK, Clone MORAG, 1:50), MSH2 (Novocastra, UK, Clone 2SD12, 1:50), MSH6 (Novocastra, UK, PU29, 1:50) and BRAF V600E (Clone VE1). Tissue sections were cut with a microtome from paraffin-embedded blocks. The cut sections were floated onto slides that were then dried in an incubator at 60°C overnight. The immunohistochemical stains have all been optimised according to departmental standard operating procedure and the manufacturer instructions for use on an automated staining machine (DAKO Autostainer Link 48, Denmark). Staining of tissue sections includes the treatment of primary antibody serum. Usage of a mouse linker resulted in increased expression of antigens. Tissue sections were washed with Tris buffered saline (TBS) at pH 7.6. The EnVision™ FLEX target Retrieval Solution, High pH, was used for antigen retrieval. The chromogen 3,3′ Diaminobenzidine hydrochloride solution (DAB, Sigma) was used, which produced a brown pigment. Meyer’s haematoxylin was the counterstain utilised. Tissue sections, in the presence of positive internal controls such as endothelial cells, stromal cells and lymphocytes, were assessed for retention of staining in tumour nuclei or an absence/loss of staining in tumour cells.

BRAF polymerase chain reaction
Tumour DNA was extracted from archived tissue blocks. Tissue sections were cut, deparaffinised, washed with ethanol, dried and placed in Protein K buffer solutions. PCR using primers targeting the BRAF V600E mutation site was undertaken according to the manufacturer’s instructions. Four mutation assays were performed for V600E/V600Ec, V600D, V600E and V600R. Both V600E and V600Ec mutations were assessed, but the V600E/EC assay did not allow for differentiation between the two. PCR was run on a Rotor-Gene Q Splex HRM instrument with fluorescence channels for Cycling Green and Cycling Yellow and analysis used Rotor-Gene Q Software version 2.1. A cycle threshold value (Ct) between 20.95 and 33 was required in order for DNA of sufficient quantity to be present for assessment according to the manufacturer’s instructions. Ct values between 35 and 45 implied that only a few amplifiable copies were detected and that mutations were identified if these were present on most copies. A Ct value of > 33 suggested that low quantities of mutations were present. Mutations were identified by calculating the difference in Ct values between the test sample and the control sample, which was provided with the purchased kit: ΔCt = (Ct value of mutation)−(Ct value of control tissue).

A value ≤ 7 was indicative of a BRAF mutation being present.

BRAF Sanger sequencing
BRAF mutational analysis by sequencing was performed on DNA extracted from archived tissue blocks which was deparaffinised, washed in ethanol, dried and placed in Protein K buffer solutions. PCR targeted the BRAF V600E mutation site and was assessed by polyacrylamide gel electrophoresis and visualised using ethidium bromide under ultraviolet light. PCR amplicons were purified using the Biospin PCR purification kit (Bior Technology Co Ltd, Hangzhou, China) according to the manufacturer’s instructions. Sequencing of PCR amplicon fragments were sequenced using the BrilliantDye™ Terminator v3.1 Cycle Sequencing Kit (NimaGen BV, Nijmegen, The Netherlands). The DNA Sequencing Clean-up Kit™ (ZYMO Research Corporation, CA, USA) was used to purify sequencing products, which then underwent electrophoresis on an Applied Biosystems 3500XL automated genetic analyser, using 50 cm capillary arrays and POP 7 polymer. Data were analysed on Geneious software. Sanger sequencing was conducted by Inqaba Biotec utilising BrilliantDye™ Terminator V3.1 cycle sequencing kit (Nimagen, Netherlands) and an ABI 3500XL Genetics analyser (Thermo Fisher Scientific®, UK) according to the manufacturer’s instructions.

MLH-1 hypermethylation using EPITYPER
MLH1 Promoter hypermethylation analysis was undertaken using MassARRAY EpiTyper analysis, by Agena Bioscience at Inqaba Biotec. This system quantitatively assessed DNA methylation. The software package had the MLH1 promoter region target sequence (−248 to −178) entered, which identified primers for the best possible DNA coverage. There was a product size of 187 base pairs, which enabled evaluation of 11 CpG islands, of which 3 CpG targets could not be assessed due to lower mass cleavage products, whilst 8 CpG sites could be evaluated. The forward primers were: AGGAGAGGAGGAGTAGCCGATT and the reverse primers were: TCTCTGGTCCCCCTCCTAAACAG. The extracted patient DNA underwent bisulphite conversion, PCR, transcription and cleavage followed by mass spectrometry of the cleaved products. In each case, the percentage of methylation was determined by comparing the signal intensity between mass signals from unmethylated and methylated template DNA. The results were depicted in an Epigram.

Statistical analysis
Data from various observations were captured on a Microsoft Excel 2013 programme (Microsoft Corp, Redmond, WA, USA). Statistical analyses were undertaken utilising STATA version 15 (StataCorp LP, College Station, TX, USA).
Categorical variables were presented as frequencies, proportions, percentages and charts. Normally distributed continuous variables were presented as means and standard deviations. Non-normally distributed continuous variables were depicted as medians and interquartile range. Cohen’s Kappa was used to assess agreement/concordance between and among BRAF IHC, PCR and sequencing as well as BRAF assessments in comparison with MLH1 methylation status.

Results
Thirty-seven out of 145 endometrial carcinomas showed loss of MLH1 staining (Figure 1). Of the 145 cases, a total of 41 MMR deficient cases were identified; 20 showed MLH1/PMS2 loss, one showed MLH1/PMS2/MSH6 loss, two cases showed MSH6/MSH2 loss whilst two cases showed loss of MSH2 and 16 (11%) showed isolated MLH1 loss. The 37 MLH1 deficient cases underwent BRAF testing by IHC, PCR and Sanger sequencing. Only one (2.7%) case showed positive cytoplasmic staining using the BRAF immunohistochemical antibody whilst 97.3% of cases were negative. (Figure 2). In contrast to IHC, PCR identified mutations in 4/37 (10.8%) of cases and of these 4 cases, 3 (75%) had V600E mutations. A single case demonstrated a V600D mutation. Only a single case showed a BRAF V600E mutation by Sanger sequencing.

Mutation-associated staining patterns detected by IHC were compared with Sanger sequencing, which is regarded as the gold standard. The sensitivity of IHC was 0% (95% CI 0), and the specificity was 97.22% (95% CI 91.92–102.52). The positive predictive value (PPV) was 0% (95% CI 0) and the negative predictive value (NPV) was 97.22% (95% CI 91.92–102.52). The overall accuracy was 94.59% and the balanced accuracy was 48.61%.

Results of BRAF PCR were compared with results obtained by BRAF Sanger sequencing. The sensitivity of PCR was 0% (95% CI 0) and the specificity was 88.89% (95% CI 78.76–99.02). The positive predictive value (PPV) was 0% (95% CI 0) and the negative predictive value (NPV) was 96.97% (95% CI 91.45–102.49). The overall accuracy was 86.47% and balanced accuracy was 44.46%.

Table 1 shows that the prevalence of BRAF mutations was 16.21% (95% CI 6.19–32.01). There was no statistically significant difference between the median age of BRAF mutation-negative women (67 years) and the median age of mutation-positive women (63 years) (p = 0.3019). There was no statistically significant association between tumour FIGO grade and BRAF result. Most (83.33%) cases with a positive BRAF mutation demonstrated FIGO grade 2 histological features.

Table 2 shows that 10.81% of cases demonstrated a BRAF mutation by PCR and that 75% of patients with a PCR detectable BRAF mutation were over 60 years of age. Furthermore, 75% of tumours with BRAF mutations demonstrated FIGO grade 2 histological features.
Table 2: Comparison of age and tumour FIGO grades of cases with mutation and those without mutation based on BRAF PCR

| Factor            | No mutation | Mutation | Total |
|-------------------|-------------|----------|-------|
|                   | N = 33      | N = 4    | N = 37 | p-value |
| Age (years)       | 89.19%      | 10.81%   | 97.30% | 0.9219  |
| < 60              | 66.15 (62.95–69.36) | 66.5 (50.56–82.44) | 66.5 (50.56–82.44) | 0.9219  |
| ≥ 60              | 66.67       | 75       | 67.57  |
| Tumour FIGO grades|             |          |        |
| FIGO grade 1      | 9           | 1        | 10     |
| FIGO grade 2      | 21          | 3        | 24     |
| FIGO grade 3      | 3           | 0        | 3      |

Table 3: Comparison of age and tumour FIGO grades of cases with mutation and those without mutation based on BRAF sequencing

| Factor            | No mutation | Mutation | Total |
|-------------------|-------------|----------|-------|
|                   | N = 36      | N = 1    | N = 37 | p-value |
| Age (years) (median) | 66.5 (63.48–69.52) | 63.64 (60.56–82.44) | 66.5 (63.48–69.52) | 0.324  |
| < 60              | 11          | 1        | 12     |
| ≥ 60              | 25          | 0        | 25     |
| Tumour FIGO grades|             |          |        |
| FIGO grade 1      | 10          | 0        | 10     |
| FIGO grade 2      | 23          | 1        | 24     |
| FIGO grade 3      | 3           | 0        | 3      |

Table 3 demonstrates that 2.70% of cases had a mutation detected by BRAF sequencing and that the only patient with a mutation was under the age of 60 and that this patient's tumour showed FIGO grade 2 histological features.

Table 4 shows the agreement between results obtained by IHC versus overall BRAF mutational status as well as sequencing and overall mutational status was 86.49%. There was a statistically significantly low, non-random concordance.17 (kappa = 0.15, p < 0.005). There was agreement of 94.59% between PCR and overall mutational status. There was statistically significant, moderate, non-random concordance17 (kappa = 0.77, p = 0.0001).

Of the six cases that showed BRAF abnormalities by IHC, PCR or Sanger sequencing, one case did not have enough DNA for methylation assessment, whilst four cases were methylated. There was no statistically significant association between methylation using Epityper and BRAF IHC, PCR and sequencing, stratified by age or by tumour FIGO grade. Similarly, there was no statistically significant association between MLH1 methylation using Epityper in relation to BRAF overall mutational status (Table 5; p = 0.6649).

**Discussion**

BRAF mutations have been implicated in 50% of colonic carcinomas and their identification suggests a sporadic event. Parsons et al.18 have reported that a tumour’s BRAF status is a more sensitive indicator of a tumour’s MMR gene mutational status as opposed to methylation.18 Studies on BRAF assessments in endometrial carcinomas have shown conflicting results. However, studies conducted in the West have shown that BRAF mutations occur too uncommonly in endometrial carcinomas and, as such, BRAF mutational analysis has not been considered as part of the work-up of endometrial carcinoma mutational status.5,19

In the present study, six BRAF mutations were identified using three test methodologies. At present, BRAF sequencing is the gold standard against which other methods are tested, but in the current study sequencing detected just a single BRAF mutation. BRAF immunohistochemical testing also only detected a single abnormality, indicating a likely underlying mutation. BRAF PCR, however, demonstrated four mutations. Of these, three were V600E mutations and a single BRAF V600D mutation was identified, which to the best of the investigator's knowledge has not been previously identified in endometrial carcinomas.
BRAF testing in our study using 3 methodologies showed discrepant results. In colorectal carcinomas, the BRAF IHC has demonstrated concordance levels with BRAF mutational assessment of over 95%. However, varying tumour types require validation of the antibody, and the antibody may not perform well in different tumours. Endometrial carcinomas may be one such tumour in which the BRAF IHC does not perform optimally. In addition, the antibody may show false-negative staining when there is low tumour volume or increased non-neoplastic cells within the tissue section. Furthermore, the antibody is only directed against V600E whilst the PCR kit used in the present study assessed other mutations such as the BRAF V600D mutation, which could not be detected immunohistochemically. The BRAF PCR kit used in this study is considered sensitive, whilst Sanger sequencing is known to have low sensitivity. A study by Zhao et al. has shown a lower sensitivity for detection of BRAF V600E by Sanger sequencing in comparison with the immunohistochemical antibody and real-time PCR. These are plausible explanations for the varied results we obtained using three different test methods. However, use of these three test methods increased the yield of BRAF mutations detected in our patient population, which is slightly higher when compared with the number of BRAF mutations detected in Western societies, but less than the number of mutations identified in an Eastern study. Whilst our study assessed BRAF mutations in only MLH1 deficient cases and may therefore not be representative of all endometrial carcinomas, the present study indicates that, like studies in the West, BRAF mutations are not commonly encountered in endometrial carcinomas in the South African population assessed and BRAF assessment is thus not useful in triaging patients with suspected Lynch syndrome.

Despite Feng et al. having identified BRAF mutations in 21% of that study’s patients with endometrial carcinomas, MLH1 methylation analysis was not performed. Feng et al. suggested a build-up of BRAF mutations in the population assessed. This suggestion has been contested by Metcalf and Spurdle as studies by other researchers on patients of Japanese descent did not yield similar results to the investigation by Feng et al. In addition, Metcalf and Spurdle have noted that there are many molecular techniques used that have high sensitivities and specificities. Thus, these tests should identify mutations regardless of the ethnicity. The differing results obtained by Feng et al. in comparison with other investigators has been ascribed by Feng et al. to dilution of tumour DNA during macrodissection. This, however, is not a likely event as Metcalf and Spurdle have stated that different researchers have detected other mutations when macrodissection was used. As such, it is not possible to be entirely certain of the reasons for the high number of BRAF mutations documented by Feng et al.

Our study shows that there is no statistically significant relationship between BRAF mutations, irrespective of the test methodology. Similarly, there is no statistically significant association between BRAF mutations and MLH1 methylation status of endometrial carcinomas, which mirrors results from studies on endometrial carcinomas from Western countries. This is in contrast to colorectal carcinomas where concordance rates of over 80% have been documented for MLH1 promoter methylation and BRAF mutations. More than 60% of immunohistochemically MLH1 deficient colorectal carcinomas that are negative for MLH1 mutation have BRAF mutations documented. Whilst loss of MLH1 immunohistochemical staining cannot separate between a somatic, sporadic tumour occurrence or a germline mutation, in colorectal carcinomas testing for MLH1 promoter hypermethylation and BRAF mutations assists in the identification of sporadically occurring tumours, suggesting that Lynch syndrome in such patients is unlikely. However, our results in endometrial carcinomas, similar to findings from Western studies, indicate that BRAF mutations are not beneficial in separating out which patients have spontaneously occurring tumours from those who may harbour germline mutations and are suspected of having Lynch syndrome.

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