Uncertainty in the Utility of Immunohistochemistry in Mismatch Repair Protein Expression in Epithelial Ovarian Cancer

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Abstract. Background: Utility of immunohistochemistry (IHC) for mismatch repair (MMR) protein expression has been demonstrated in colorectal cancer but remains incompletely defined in ovarian cancer. We evaluated MMR protein expression in three population-based samples of epithelial ovarian cancers. Materials and Methods: IHC staining was performed on full-section (FS) or tissue microarray (TMA) slides for MLH1, MSH2, and MSH6 expression. Results: Out of 487 cases, 147 and 340 were performed through FS and TMA, respectively. Overall, Loss of Expression (LoE) of at least one MMR protein was observed in 12.7% based on an expression score of ≤3 (on a scale of 9). Notably, LoE was significantly higher in TMAs (17.9%) compared to FS cases (0.7%) (p<0.001). Conclusion: A substantial proportion of epithelial ovarian cancers have a loss of MMR protein expression. Protein expression results vary significantly by the tissue sampling methodology utilized, raising concerns about the clinical utility of this test for ovarian tumors. Ovarian cancer ranks fifth in both cancer incidence and cancer mortality in U.S. women, having one of the highest mortality rates among gynecological cancers (1). Defects in the mismatch repair (MMR) pathway, one of the best defined molecular pathways involved in both inherited and sporadic cancer pathogenesis, (2) may be etiologically important in a substantial proportion of ovarian cancers (3). Established methods to classify tumors as MMR-deficient cancers include: 1) immunohistochemistry (IHC) to measure loss of MMR protein expression; and 2) microsatellite instability analysis to identify those with a microsatellite instability-high (MSI-H) phenotype.

The assessment of MMR protein expression is well-accepted for identification of Hereditary Non-Polyposis Colorectal Cancer (HNPPC)-associated cancers (i.e., cancers of the colorectum, endometrium, stomach, ovaries, urinary tract, other gastrointestinal sites and brain) (4). In fact, loss of MMR protein expression may help to identify those with germline MMR gene mutations, which in turn may provide individuals with an opportunity for cancer prevention through colorectal, endometrial and ovarian cancer risk management options, as outlined in the NCCN guidelines (5). In addition, it provides at-risk relatives the opportunity to prevent cancers through increased cancer screening and/or prophylactic surgeries (6, 7). Furthermore, MMR deficiency has the potential to identify a specific pathway to tumorigenesis, which could have implications to diagnosis and treatment.

Most studies have evaluated MMR deficiency in colorectal and endometrial cancers. The few studies of MMR deficiency in ovarian cancer have either only focused on IHC in a small series of cases, (8-10) or only among cases identified as microsatellite instability-high (MSI-H) (11, 12). Moreover, the methods to define MMR deficiency have varied, with some performed on paraffin-embedded full-section slides (8) and others based on tissue microarray...
(TMA) slides (9, 10). Additionally, the scoring system by which loss of expression (LoE) of proteins on IHC was defined varies across studies, and has been only partly been described in many instances. Furthermore, prior reports include limited characterization of these tumors on demographic and clinical variables. There are data to suggest that MMR-deficient tumors tend to occur prior to age 50, particularly those associated with germline mutations in the MMR genes (13). Furthermore, Sood et al. reported that MMR-deficient ovarian tumors (based on MSI-H status) had a mean age at diagnosis that was five years earlier than ovarian tumors without MSI-H (14). Another finding observed in most studies is overrepresentation of non-serous histologies, such as endometrioid, mucinous and clear-cell subtypes, in those with MMR-deficient ovarian tumors (3, 15). Although a recent meta-analysis estimated a frequency of MMR deficiency of approximately 10% based on IHC and/or MSI results, (3) the data remain insufficient to inform the clinical utility of this test for ovarian cancer.

The purpose of the current study was to quantify the frequency of ovarian tumors with MMR deficiency through IHC for MMR protein expression through use of a mix of full-section and tissue microarray (TMA) slides, as well as demographic and clinical predictors of the loss of expression phenotype. A secondary objective was to compare full-section and TMA-based results, to explore the clinical utility of IHC for MMR protein expression.

**Materials and Methods**

**Participants.** Participants for this study were drawn from three population-based studies of epithelial ovarian cancer: the Familial Ovarian Tumor Study (FOTS) in Toronto, (16) the Tampa Bay Ovarian Cancer Study (TBOCS) at the Moffitt Cancer Center, (17) and the North Carolina Ovarian Cancer Study (NCOCS) at Duke University, (18) with details about study design, populations, and data collection methods published previously. Briefly, FOTS cases were identified through monitoring of pathology reports submitted to the population-based Ontario Cancer Registry for province-wide recruitment. TBOCS cases were recruited through a rapid case ascertainment mechanism in the two most populous counties in the Tampa Bay region. NCOCS cases were identified through a rapid case ascertainment mechanism in a 48-county region located in the central portion of North Carolina. The study protocol was approved by the institutional review board at each center, and written informed consent was obtained from all participants.

Eligibility criteria for study enrollment were defined as incident, pathologically confirmed primary epithelial ovarian cancer, either borderline or invasive, aged 20 years and above. Through each of the studies, data collection included: administration of a questionnaire, collection of demographic, clinical, and family history information, and review of medical records for histopathology of the ovarian tumor. Formalin-fixed paraffin-embedded epithelial ovarian tumor blocks were obtained from the hospital at which the participant had their surgery for 487 participants: 142 from Duke, 219 from Moffitt, and 126 from Toronto.

**Construction of TMA.** One representative H&E stained full-section slide of each tumor was reviewed by expert pathologists (DC, SVN) to confirm the diagnosis. For all Duke cases and 5 Moffitt cases, full sections representative of the tumor were reviewed and used for the immuno-histochemical analysis. For the remaining 214 Moffitt cases and all Toronto cases, the pathologists selected 3 representative areas of tumor to be included on the TMA blocks.

The TMA blocks were constructed using the same protocol for the FOTS patients (based in Toronto) and the TBOCS patients (based at Moffitt) with a precision instrument (Chemicon model ATA-100, Chemicon Int’l, Temecula, CA, USA) as previously described (19). For each case, three replicate cores (1 mm in size for Moffitt TMA cases and 0.6 mm in size for Toronto cases) were sampled from the donor tissue block and placed side-by-side on a separate recipient block. Normal control tissue (fallopian tissue) was included in the block. A heated glass slide was used to even the surface of the recipient block. Sample tracking was based on coordinate positions for each tissue core in the TMA recipient block; 4μm sections were transferred onto separate TMA slides for IHC staining of all of the three MMR proteins under investigation (hMLH1, hMSH2, and hMSH6).

**Immunohistochemistry for MMR proteins.** De-paraffinized, formalin-fixed paraffin-embedded tissues were microwaved in 1X EDTA (Chemicon Int’l, Temecula, CA) (hMSH2) or BORG Decloaker (BioCare Medical, Concord, CA) (hMLH1 and hMSH6), cooled at room temperature for 20 min, rinsed with deionized water and placed in TBS/Tween for 5 min. Immunostaining was carried out on the Dako Autostainer using the Vector Elite Mouse IgG – HRP detection kit (Vector Laboratories, Burlingame, CA) following avidin/biotin blocking (Vector Laboratories). Slides were incubated in mouse monoclonal hMLH1 (clone G168-15, BioCare Medical, Concord, CA) at 1:40 or hMSH6 (clone BC/44, BioCare, Concord, CA) at 1:70 overnight at 4°C or hMSH2 (Clone FE11, Zymed/Invitrogen, Carlsbad, CA) at 1:200 for 30 min at room temperature. For overnight incubations, slides were removed from the autostainer, placed in a humid chamber in the refrigerator, and returned to the autostainer the following day. 3,3’-Diaminobenzidine (Dako, Carpinteria, CA) was the chromogen. Slides were counterstained with modified Mayer’s hematoxylin, dehydrated through ascending grades of ethanol, cleared with xylene and mounted with resinous mounting medium.

**Immunohistochemistry analysis.** Loss of MMR expression was defined as absence of detectable nuclear staining of tumor cells in the presence of retained nuclear staining in lymphocytes and/or in non-neoplastic epithelial or stromal cells, which served as internal positive controls. The stained tissue full-sections slides and TMA cores were examined by senior board-certified pathologists (DC, SVN). Stainings were classified based on nuclear staining intensity and distribution using a semi-quantitative ordinal scoring system to generate a combined expression score. The intensity and staining were scored on a scale of 0-3. Specifically, the intensity score was graded based on least intense (score of 0) to most intense (score of 3) and the staining was graded based on the percentage of positive cells as follows: 0(0%), 1(1-33%), 2(34-66%) and 3(67-100%). The product of the intensity and staining was used as the final score. The score was calculated for each of the 3 cores of each sample and the mean of the 3 scores was used as the final score. The final scores were classified as: 0, negative, 1-3, weak, 4-6, moderate and 7-9, strong.
Data collection. All participants completed a study questionnaire and interview, by which demographic and family history variables were collected. Family history information included collection of all ages and types of cancer diagnosed in first, second and third degree relatives. Medical records were retrieved on all participants to abstract information on tumor histology. In addition, information on date of diagnosis (based on pathology report) and date of study enrollment (based on date consent form signed) were collected, in order to determine time between diagnosis and enrollment (calculated in days).

Statistical analyses. Differences in demographic, clinical, and pathological variables across the specimen types (full section, 1.0 mm TMA core, 0.6 mm TMA core) were evaluated using Kruskal-Wallis tests for continuous variables and Pearson Chi-square exact tests based on Monte Carlo estimation for categorical variables. Frequencies/proportions of LoE were calculated overall and also according to specimen type, including LoE as defined by cut-off scores of 3 and below and 4 and below. The 95% confidence intervals for the proportions of LoE were calculated using the Wilson confidence (score) limits method. Further, demographic and clinical variables were compared between LoE and no LoE patient subgroups using Wilcoxon rank sum tests and Pearson Chi-Square exact tests. For this analysis, full section cases were excluded due to the limited sample size (as only 1/147 of these samples had LoE). All reported p-values are two-sided. All analyses were carried out with R version 2.13.1.

Results

The demographic, clinical, and pathological features of the participants are summarized in Table I. Tumor tissues were examined on a total of 487 participants with a mean age of 56.1 years. Proportions of invasive and borderline cases were 85% and 15%, respectively. Of the invasive cases, 61.8% were of serous histology. Family history of an HNPCC-associated cancer was reported by 34.5% of participants. The time-to-study enrollment was highest in the TMA cases with 0.6 mm cores (i.e., which represents cases from Toronto), compared to the full-section and 1 mm core cases which had comparable median time from diagnosis to enrollment. Similarly, for the full section cases, there were a higher proportion of borderline tumors, likely attributable to survival bias resulting from the higher median time from diagnosis to enrollment.

The frequency of LoE, defined as a score of 3 and below on samples based on full-section slides, 1 mm TMA cores and 0.6 mm TMA cores was 0.7%, 15.9% and 21.4%, respectively (Table II). When using a cut-off score of 4, all LoE frequencies were higher for all specimen types, as expected (i.e., 13.6%, 27.5%, and 26.2%, respectively). The estimated frequencies were statistically significantly different between full-sections, 1 mm TMA and 0.6 mm TMA regardless of cut point used.

Evaluation of demographic and clinical variables was limited to TMA-based cases (n=340) since analysis based on full-section specimens yielded only 1 case with LoE. Overall, cases with LoE were similar to cases without LoE for all variables except one: histological subtype. In particular, subgroup analyses of histological subtypes in those with invasive tumors revealed an association of tumors with LoE and non-serous subtypes (p=0.003), with the highest frequency seen in those with mucinous cancers (38.9%), followed by clear cell (35.3%) and endometrioid (23.1%) subtypes.
Table II. IHC results of MMR expression in epithelial ovarian cancer by specimen type.

<table>
<thead>
<tr>
<th>LoE score cut-off value</th>
<th>Overall (n=487)</th>
<th>Full-sections (n=147)</th>
<th>1-mm cores (n=214)</th>
<th>0.6-mm cores (n=126)</th>
<th>p-Value</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoE (n (%))</td>
<td>95% CI</td>
<td>LoE (n (%))</td>
<td>LoE (n (%))</td>
<td>LoE (n (%))</td>
<td>(1 mm vs. 0.6 mm)</td>
<td></td>
</tr>
<tr>
<td>LoE defined as &lt;3</td>
<td>62 (12.7)</td>
<td>10.0-16.1</td>
<td>1 (0.7)</td>
<td>34 (15.9)</td>
<td>27 (21.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LoE defined as &lt;4</td>
<td>112 (23.0)</td>
<td>9.4-27.0</td>
<td>20 (13.6)</td>
<td>59 (27.5)</td>
<td>33 (26.2)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Discussion

In this study we evaluated MLH1, MSH2, and MSH6 protein expression through IHC in 472 samples of ovarian carcinomas of diverse histological subtypes. Our findings indicate an overall frequency of loss of MMR protein expression of 12.7%. Furthermore, the frequency of LoE observed varied by specimen type, with a lower overall frequency of LoE in full-section slides (0.7%) compared to TMA slides (15.9% and 21.4% for 1 mm and 0.6 mm cores, respectively), which suggest that specimen type is important to consider when assessing IHC in ovarian samples. Finally, our data suggest that LoE may be more common in ovarian epithelial tumors with non-serous histologies.

Our overall TMA-based estimates (17.9%) are higher than that reported in the few studies, to date, that have evaluated loss of MMR protein expression based on IHC in unselected invasive epithelial ovarian carcinomas, whereas our full-section estimates (0.7%) are lower (8-10). The study by Domanska et al. (8) represents the only report of IHC on full section ovarian cancer slides; loss of MLH1, MSH2, MSH6 and PMS2 expression was 6.1% in patients age 40 or below at diagnosis. Two studies have performed IHC on TMA sections. Malander et al. (9) evaluated MLH1, MSH2, MSH6 and PMS2 and noted loss of protein expression in 2.3% (3/128) of cases. Rosen et al. (10) reported a loss of expression of MLH1 and MSH2 based on TMA sections in 2.2% (7/322) of unselected ovarian cancers. More recently, a meta-analysis estimated a frequency of MMR deficiency of approximately 10% based on IHC and/or MSI results in ovarian cancers (3).

Although our study is the largest, to date, of MMR protein deficiency in ovarian cancer based on IHC, it is also the first multi-center study to include both TMAs and full-sections, thus the lack of consistency in MMR frequency found in our study could not have been detected in prior efforts. Furthermore, the limited details on IHC methodology (such as detailed protocol, equipment and antibodies, and the scoring system) in prior reports preclude our ability to fully compare our results with prior studies. Of note, the LoE frequency varied based on whether a cut-off score of 3 versus 4 was used, suggesting it is imperative to report scoring methodologies in published reports to allow cross comparisons between studies.

Validation of the use of TMA for MMR protein expression has been evaluated in a single colorectal cancer study in which IHC for MLH1 and MSH2 was conducted in 263 colorectal cases through TMA (3 cores per case) and full-section. Results indicated comparable frequencies of 98.8% and 99.2%, respectively (20). In ovarian cancers, validation of TMA technology has been performed on several markers, including p53, Ki-67, estrogen and progesterone receptors, among others, (21, 22) however to our knowledge there are no published studies that have validated IHC for MMR proteins expression in ovarian cancer. Specifically, the study by Rosen et al. represents the only published report in which full-sections and TMAs were compared in high-grade serous ovarian cancers through IHC studies of Ki-67, estrogen receptors and p53 and correlation co-efficients were reported as 0.86, 0.93, and 0.82, respectively (23). In general, correlation co-efficients of greater than 0.8 are considered evidence of excellent correlation. In a subsequent study, Hecht et al. performed IHC for Ki-67, estrogen receptors, and p53 among others using triplicate core samples from 174 epithelial ovarian cancers and demonstrated high intraclass correlation between the cores (21). Ultimately, there have been studies to validate the use of TMAs for MMR protein expression in colorectal cancer as well as studies to validate the use of TMAs to evaluate protein expression in ovarian cancers (which have not included MMR proteins). Consequently, studies to evaluate MMR protein expression in ovarian cancers using either TMA or full-section slides have been conducted. Our study differs from prior efforts as we used a combination of TMAs and full-section slides to evaluate MMR protein expression in ovarian cancers. Based on published reports, we presumed TMA and full-section cases were equivalent, however our results pertaining to MMR expression suggest they may not be directly comparable due to differences observed by specimen type. These findings suggest that validity of TMA may vary by antigen, and clearly demonstrate the need for large scale studies to validate the use of IHC for MMR protein expression specifically in ovarian cancers, prior to their use in the clinical setting. Moreover, in an effort to further clarify these differences, tumor samples on 19 participants in the study population were evaluated through both full-section and
TMA slides for expression of three MMR proteins based on IHC. Results demonstrated poor correlation of full-section and TMA-based samples, with a LIN correlation coefficient of 0.4, 0.3 and 0.7 for MLH1, MSH2 and MSH6, respectively (data not shown).

Possible reasons for the discrepancy observed by specimen type include the large size at diagnosis of ovarian tumors compared to colorectal tumors, which may contribute to their heterogeneity as they may be composed of different clonal populations exhibiting different MMR reactivity. Another possible explanation is that the fixative does not adequately penetrate the large blocks of cancer, typical of ovarian cancer, which may impact IHC for some proteins more than others. Interestingly, the frequency of LoE inversely correlated with the size of surface area of the tissue examined (0.7% in full-sections (usually several mm in diameter) vs. 15.9% in TMA cores of 1 mm in diameter vs. 21.4% in TMA cores measuring 0.6 mm in diameter). It is possible that when evaluating larger size tissues, there is a higher likelihood of identifying even a small area strongly staining for the evaluated marker, thus potentially leading to a lower frequency of LoE. Conversely, when a small size sample of a large tumor is evaluated, a negative stain will not completely exclude the possibility that a non-sampled area of the same tumor be positive. Finally, another possibility considered was whether discrepancy in results may have occurred due to considerable differences between the three study sites. However, comparison of demographic variables between study participants did not suggest any underlying differences in the study populations at the three sites to account for our findings. In fact, all three sites encompassed primarily a Caucasian population and each study was population-based, thus collected specimens from multiple hospitals within their study area. Ultimately, future efforts focused on differences between full section and TMA slides are needed on a larger range of antibodies, to determine their usefulness for Her2 (26). Ultimately, the implementation of similar guidelines for ovarian cancer specimens will improve the immunohistochemical evaluation of these tumors, and will improve our ability to better determine the basis of discrepant results, as they arise. Finally, IHC was limited to the three more commonly stained proteins, MLH1, MSH2, and MSH6, at the time at which testing was done. PMS2 was not done, as it has more recently been included in routine IHC analyses.

In conclusion, our study suggests that a significant portion of human ovarian cancers exhibit defects in the mismatch repair pathway, proposing this pathway as a potential contributor to the development of epithelial ovarian cancers. The results also indicate that immunohistochemical techniques for the assessment of MMR proteins deficiency may have limited diagnostic accuracy in ovarian cancer and require further validation studies to assess their utility. Future efforts focused on inter and intra-institutional standardization of IHC methodologies is crucial for the successful utilization of this technique for clinical usage in ovarian tumors.

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References

Table III. Loss of MMR protein expression (LoE) in epithelial ovarian cancer cases with TMA slides.

<table>
<thead>
<tr>
<th>Proportions in TMA slides - n (%)</th>
<th>Row Total, n</th>
<th>LoE</th>
<th>No LoE</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age - Mean (SD)</td>
<td>340</td>
<td>61/340 (17.9)</td>
<td>279/340 (82.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Histology</td>
<td>340</td>
<td>56.4 (13.1)</td>
<td>56.5 (11.8)</td>
<td>0.927</td>
</tr>
<tr>
<td>Borderline, n (%)</td>
<td>39</td>
<td>6 (1.8)</td>
<td>33 (9.7)</td>
<td>0.821</td>
</tr>
<tr>
<td>Invasive, n (%)</td>
<td>301</td>
<td>55 (16.2)</td>
<td>246 (72.4)</td>
<td></td>
</tr>
<tr>
<td>Serous, n (%)</td>
<td>178</td>
<td>24 (9.1)</td>
<td>154 (58.1)</td>
<td>0.003**</td>
</tr>
<tr>
<td>Non-Serous, n (%)</td>
<td>87</td>
<td>25 (9.4)</td>
<td>62 (23.4)</td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>17</td>
<td>6 (6.9)</td>
<td>11 (12.6)</td>
<td>0.364</td>
</tr>
<tr>
<td>Endometriod</td>
<td>52</td>
<td>12 (13.8)</td>
<td>40 (46.0)</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>18</td>
<td>7 (8.0)</td>
<td>11 (12.6)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>36</td>
<td>6 (16.7)</td>
<td>30 (83.3)</td>
<td>-</td>
</tr>
</tbody>
</table>

Family history
Relatives with colorectal cancer (CRC)
\# (%) of subjects with relatives with CRC | 64 | 14 (4.1) | 50 (14.7) | 0.466 |
\# (%) of subjects with no relatives with CRC | 276 | 47 (13.8) | 229 (67.4) |        |
Relatives with endometrial cancer (EC)
\# (%) of subjects with relatives with EC | 19 | 3 (0.9) | 16 (4.7) | 0.955* |
\# (%) of subjects with no relatives with EC | 321 | 58 (17.1) | 263 (77.4) |        |
Relatives with any HNPCC cancers
\# (%) of subjects with relatives with any HNPCC cancers | 116 | 23 (6.8) | 93 (27.4) | 0.544 |
\# (%) of subjects with no relatives with any HNPCC cancers | 224 | 38 (11.2) | 186 (54.7) |        |

** Test between Serous and Non Serous, excluding other.

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