Sampling Strategies for Tissue Microarrays to Evaluate Biomarkers in Ovarian Cancer

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Abstract

Introduction: Tissue microarrays (TMA) enable rapid analysis of biomarkers in large-scale studies involving archival tumor specimens, however, their utility in heterogeneous tumors such as ovarian cancer is limited.

Methods: In this study, immunohistochemical analysis was done on TMAs comprised of epithelial ovarian cancer (EOC) to estimate the prevalence of loss of expression of three mismatch repair proteins. TMAs were initially created using cores sampled from the center of donor tissue blocks from 59 EOC cases. Full sections were subsequently created and levels of expression were compared between tissues sampled from the central portion versus the periphery. Follow-up analyses were done by obtaining cores from the periphery of up to five additional donor blocks per case. A linear mixed model for each protein was used to investigate differences between results from the initial and follow-up blocks.

Results: In the original TMAs created using centrally sampled cores, loss of mismatch repair expression was noted in 17 (29%) of the 59 cases. By comparison, analyses from peripherally sampled cores revealed loss of expression in only 6 of these 17 cases. For each protein, significant differences (P < 0.05) were detected between results from the initial donor block and the majority of the follow-up blocks.

Conclusions: Our investigations, based on EOC, suggest that sampling variability in protein expression may result when TMAs are used. Thus, at least for EOC, it is important to preferentially sample from the periphery of tumor blocks where exposure to tissue fixatives is optimal. (Cancer Epidemiol Biomarkers Prev 2009;18(1):28–34)
Ovarian cancers, diagnosed in <22,000 women per year in the United States, cause more deaths than any other gynecologic malignancy (22). Although an understanding of the molecular pathways underlying ovarian tumorigenesis is currently lacking, there is evidence that a proportion of cases are due to defects in the mismatch repair (MMR) genes, primarily MLH1, MSH2, and MSH6 (23-29). Mutations in these genes lead to hereditary nonpolyposis colorectal cancer syndrome (30, 31), an inherited cancer-predisposing condition. Immunohistochemical analysis has been a useful strategy for investigating MMR defects in colorectal cancer (32, 33), although there is limited information as to its utility in other hereditary nonpolyposis colorectal cancer-associated cancers, such as ovarian cancer (34-37). Thus, evaluation of the role of MMR deficiency in the pathogenesis of EOC is of considerable interest (36-39). To date, investigation of MMR protein expression in EOC has yielded frequency estimates of loss of expression between 2% and 10% (34-37). To improve on the precision of these estimates using TMAs, we did a validation study.

Materials and Methods

Participants and Tissue Samples. EOC tumor blocks used in the current investigation were obtained from participants in the Tampa Bay Ovarian Cancer Study, a population-based study of incident EOC in a heavily populated two-county region of west central Florida. The study was approved by the Institutional Review Board of the University of South Florida. Further details about study design, population, and data collection methods have been published previously (40). Briefly, cases included 232 women ages 18 to 80 with histologically confirmed invasive or borderline EOC diagnosed between December 13, 2000 and September 30, 2003, on whom paraffin-embedded tumor specimens from 59 of the 232 subjects were sampled from the center of the donor tissue block of representative cores obtained from the periphery of up to five additional donor tissue blocks (triplicate cores per block) determined the number of retained nuclear staining in lymphocytes and/or in nonneoplastic epithelial or stromal cells, which served as internal positive controls. Two pathologists with expertise in ovarian pathology (S. Narod and N. Valkov) independently reviewed all stainings. Stainings were classified based on nuclear staining intensity and distribution using a semiquantitative ordinal scoring system in which a combined expression score of 0 represents total absence of expression and a combined expression score of 9 represents total presence of expression. After taking into account the expected level of immunoreactivity, specimen size, the amount of target antigen in the specimen, and clinical appropriateness (18), the study pathologist defined cases with reduced or absent staining as having a mean core expression score of ≤4.

Construction of TMAs. A TMA was constructed from paraffin-embedded tumor specimens from 59 of the original 232 subjects. Archived, formalin-fixed, paraffin-embedded EOC tissue blocks that had been stored at room temperature were used. H&E-stained full sections were reviewed to select representative areas of tumor in the center of an initial donor block from which cores were acquired for the microarray. The TMA blocks were constructed with a precision instrument (Chemicon) at ascending grades of ethanol, cleared with xylene, and mounted with resinous mounting medium. Immunohistochemical Staining for MMR Proteins. Deparaffinized, formalin-fixed paraffin-embedded tissues were microwaved in 1× EDTA (hMSH2; Chemicon) or Biorad Decloaker (hMLH1 and hMSH6; BioCare Medical), 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) following avidin/biotin blocking (Vector Laboratories). Slides were incubated in mouse monoclonal hMLH1 (clone G168-15; BioCare Medical) at 1:40 or hMSH6 (clone BC/44; BioCare Medical) at 1:70 overnight at 4°C or hMSH2 (Clone FE11; Zymed/Invitrogen) 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) 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.

Loss of MMR expression was defined as the absence of detectable nuclear staining of tumor cells in the presence of retained nuclear staining in lymphocytes and/or in nonneoplastic epithelial or stromal cells, which served as internal positive controls. Two pathologists with expertise in ovarian pathology (S. Narod and N. Valkov) independently reviewed all stainings. Stainings were classified based on nuclear staining intensity and distribution using a semiquantitative ordinal scoring system in which a combined expression score of 0 represents total absence of expression and a combined expression score of 9 represents total presence of expression. After taking into account the expected level of immunoreactivity, specimen size, the amount of target antigen in the specimen, and clinical appropriateness (18), the study pathologist defined cases with reduced or absent staining as having a mean core expression score of ≤4.

Creation of Regular Tumor Sections and Subsequent Follow-up Analysis. For the 17 cases showing reduced or absent staining, full tumor sections were created from the respective donor paraffin block (from which the cores were derived) and were subsequently stained in order to evaluate a larger tumor area. Follow-up analyses for these cases were done by creating a new TMA comprised of representative cores obtained from the periphery of a part additional donor tissue blocks (triplicate cores per block) per case. The number of blocks available per case (ranging from 1 to 20) determined the number of additional donor tissue blocks sampled in the follow-up analysis. Additionally, for 5 of the 42 cases in which staining was present, full sections were created from the original donor block and were stained for immunohistochemical expression of MMR proteins.

Statistical Analysis. Descriptive statistics including graphical illustrations were generated for each protein, and summary statistics and distributions of expression scores were examined by case and by block. The distribution of protein values by block was analyzed using the Anderson-Darling statistic to gauge the need for data transformations. General linear mixed effects model were used to investigate differences between
immunohistochemical expression scores from the initial donor block and follow-up blocks for each protein, with the main interest variable being the block (41, 42), whereas adjusting for potential confounding factors. In the generalized linear mixed effects model, the variable for cases nested within the core was included as a random intercept. The correlation among the multiple observations within the same core (also nested within each block) was accounted for, assuming the compound symmetry correlation structure in the model. We applied the small-sample inference method for the fixed effects to the generalized linear mixed effects model, as proposed by Kenward and Roger (43), to adjust for the small number of cases. Several covariates were included in the initial model: tumor age (year), tumor size, number of cores, and number of blocks. Specific contrasts comparing the expression score for the initial block with the score for each subsequent block were tested for each protein model, using the F test statistic. The final model included the fixed effect of the block and the random effect of intercept. All tests were two-sided and claimed statistically significant at the level of 5%. No adjustment for multiple comparisons was applied, as the nature of this investigation was hypothesis-generating. SAS software was used for all statistical analyses (SAS Institute, version 9.1, SAS Institute, Inc.).

Results

Initial immunohistochemical analysis of the 59 cases for expression of three MMR proteins (i.e., MLH1, MSH2, and MSH6) revealed loss of expression of at least one MMR protein (characterized by a mean expression score ≤4) for 17 of the 59 (29%) cases. The tumor samples used to create the TMAs for these cases were sampled from the central portion of the respective original donor block. Characteristics of the tumors under study are shown in Table 1. Of the 17 cases in which there was initial loss of expression, the mean age of these tumor blocks was 59.5 months (range, 45-74; SD, 2.6). The mean number of blocks sampled upon follow-up was 3.6 (range, 1-5; SD, 0.4). The mean number of cores sampled upon follow-up was 10.8 (range, 3.15; SD, 1.1).

To evaluate the potential that the location of the core to create the TMA influenced the staining results, we compared staining from the same block, but on cores taken from the periphery of the paraffin-embedded tumor tissue, as compared with those taken from the center. Results of staining of full sections showed that 11 of the 17 tumor sections lacked expression in the central portion, but positive expression in the periphery. Tumor sections for the other six cases revealed a lack of expression in both the center and periphery. Additional analyses were done on a subset of the 42 cases that showed positive expression on the initial TMA, all of which were sampled centrally. Full sections were created on 5 of the 42 cases for immunohistochemical analysis, and results showed consistent protein expression in both the center and periphery. Additionally, a subset of 19 randomly selected cases not used in the TMA were stained with vimentin, which is an antigen particularly sensitive to inappropriate fixation (44). Of these cases, 16 showed positive vimentin staining, demonstrating adequate fixation. In three cases, the vimentin stain was negative, suggesting inadequate fixation.

In order to clarify the initial immunohistochemistry results, follow-up analyses were done with the construction of a new TMA comprised of peripheral cores created from the 17 cases. The results of immunohistochemical expression for the three MMR proteins showed a loss of expression in the same six cases (i.e., 10% of the total sample) that had lacked both central and peripheral protein expression on full sections. Sample images comparing MLH1 expression from cores sampled centrally versus peripherally from a mucinous adenocarcinoma are illustrated in Fig. 1, and these observations were consistently seen in those cases with initial loss of MMR protein expression when sampled centrally, which upon preferential peripheral sampling, indicated the

<table>
<thead>
<tr>
<th>Histologic subtype</th>
<th>Total (n = 59)</th>
<th>Initial loss of expression (n = 17)</th>
<th>No initial loss of expression (n = 42)</th>
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<tr>
<td></td>
<td>No. of cases (%)</td>
<td>No. of cases (%)</td>
<td>No. of cases (%)</td>
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<tr>
<td>Serous</td>
<td>26 (44.0)</td>
<td>5 (29)</td>
<td>21 (50)</td>
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<td>Endometrioid</td>
<td>11 (18.6)</td>
<td>5 (29)</td>
<td>6 (14)</td>
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<tr>
<td>Mucinous</td>
<td>8 (13.6)</td>
<td>3 (18)</td>
<td>5 (12)</td>
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<tr>
<td>Mixed cell, NOS</td>
<td>8 (13.6)</td>
<td>3 (18)</td>
<td>5 (12)</td>
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<tr>
<td>Adenocarcinoma, NOS</td>
<td>2 (3.4)</td>
<td>1 (6)</td>
<td>1 (2)</td>
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<tr>
<td>Clear cell</td>
<td>1 (1.7)</td>
<td>0</td>
<td>1 (2)</td>
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<td>16 (27.1)</td>
<td>5 (29)</td>
<td>11 (26)</td>
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<tr>
<td>II</td>
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<td>10 (59)</td>
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presence of expression. These results were not attributed to an edge effect, as the criteria used to distinguish an edge effect from true positive staining is that edge effects generally involve all components of the tissue affected, and thus, would not be specifically localized to the nuclei, in contrast with the findings illustrated in Fig. 1. Statistical analyses were done to compare the results of the initial and follow-up immunohistochemical analyses. For each of the three MMR proteins, the overall mean expression score was lower for the initially centrally sampled block as compared with the overall mean expression score for each of the peripherally sampled follow-up blocks. Specifically, the difference in results between the initial donor block and follow-up blocks for each protein were statistically significant for MLH1 (all \( P < 0.001 \)) and MSH2 (all \( P < 0.001 \)) in all cases and in three of five comparisons for MSH6 (at the \( P = 0.001 \) level). Thus, there were lower overall mean expression scores in the initially centrally sampled block as compared with the overall mean expression score for each of the peripherally sampled follow-up blocks. Initially, each generalized linear mixed effects model was fitted to account for potential confounding factors such as tumor size, tumor block age, and the number of blocks sampled. None of these variables altered the overall mean expression score across blocks in the models. Box plots were generated to visually portray the difference in expression scores when comparing central versus peripheral sampling from the initial versus follow-up blocks, respectively (Fig. 2). The observed differences could not be accounted for by tumor size, tumor block age, the number of blocks sampled, or the number of cores punched per block, for any of the three proteins examined.

### Discussion

The results of the current study illustrate the potential effects that the tissue sampling strategy can have on biomarker studies when TMAs are used as the primary platform for protein expression analysis. Specifically, our results indicate that peripheral, rather than central, sampling of tumor blocks has the potential to yield more accurate results regarding the presence of MMR protein expression in a given EOC tumor. The impetus for this study stemmed from findings obtained during the completion of a larger population-based study investigating MMR protein expression in EOC. In that previous investigation, TMA platforms were used to determine MMR expression levels and the observed loss of expression estimates were much higher than the expected 2% to 10% reported in the literature (34-37). Motivated by this discrepancy, we sought to explore possible explanations for this difference. Further investigations subsequently implicated that tissue core sampling strategy within the tumor specimens during the creation of TMAs was the likely source of the difference. In the current study, improper tissue fixation is the most likely etiology of the discrepant finding between central and peripheral sampling. Previous investigations have shown that formaldehyde-based fixation of tumor tissue (2, 45, 46) is a factor when interpreting immunohistochemical results. Cross-linking of proteins is one of the most critical molecular changes induced by formalin, and the ideal fixation time for a 5-μm-thick tissue block is 12 to 24 h (46). Underfixation occurs when formalin diffuses slowly into tissues, resulting in strong staining near the periphery of a tumor block and less staining in the center (45), where fixation is poor, due to inadequate...
preservation. Tissue fixation is one of the least controlled phases of the immunohistochemical staining process because fixation conditions (i.e., time to fixation, total fixation time, and rate of fixative penetration in tissues of different types and thicknesses) can vary by specimen and institution (47, 48). Tissue underfixation as the cause of discrepant TMA results has previously been reported in prostate cancer (45), although there are no such reports in EOC. Our findings highlight the need to consider the effect of improper fixation on biomarker expression in TMA-based studies. Previous investigations have shown that tumors should be fixed within 30 minutes of surgical removal of the tissue, as delayed fixation causes increased proteolytic degradation. Depending on the antigen, this may lead to irreversible weak or absent staining (46). The specimens should remain in the fixative for at least 12 hours to avoid underfixation, yet no longer than 24 hours to minimize overfixation (46).

Another source of variation of immunohistochemical results is the presence of tissue heterogeneity, which affects the extent to which TMA-derived cores are representative of the parent tumor from which they are derived. For example, colorectal cancers are often large and highly heterogeneous with marked stromal areas between glandular structures. This results in an increased chance that a core biopsy may miss tumor cell–rich regions. In contrast, in the case of thyroid malignancies in which tumors consist of more homogeneous regions packed with cancer-rich cells, there is less concern that a sampled core may misrepresent the tumor as a whole (8). This concern regarding tissue heterogeneity is not unique to cores, however, because conventional tumor sections also represent a small fraction of the volume of most tumors (4). Validation studies have compared molecular expression data derived from cores and conventional full sections, and have shown high concordance between the methods used (4, 7, 12, 49), although the concordance varies according to tumor type and the respective degree of heterogeneity. The degree of heterogeneity is influenced by intratumor levels of ischemia and/or proximity to vessels or stroma, resulting in effects on delivery of chemokines, growth factors, or other modulators (50).

Heterogeneity is frequently seen in EOCs, which are typically large in size (51-53). Furthermore, as with other tumor types, there is biological variability within and between individual EOC tumor blocks (17). However, to our knowledge, no studies to date have compared immunohistochemistry results from core-section–derived versus full-section–derived methods when using EOC specimens. In the current study, an internal validation study was completed prior to using TMAs in this investigation, which involved the comparison of full-section versus TMA-derived immunohistochemical results of a small subset of EOC cases, and findings showed concordances of 95%, 90%, and 75% for MLH1, MSH2, and MSH6, respectively (data not shown). Although reassuring, large validation studies are warranted when investigating biomarkers in heterogeneous tumors like EOC, as the use of TMAs may be limited due to inadequate sampling of representative tumor tissue due to tumor heterogeneity. Overall, the higher the degree of tumor heterogeneity, the less likely TMAs may be to adequately represent the tumor of origin.

Although staining artifacts at the edge of tissue sections, referred to as leading edge effects, are a known phenomenon in immunohistochemistry (8, 46), such artifacts are unlikely to be responsible for the discrepant biomarker expression scores when sampling from the periphery versus the center of the individual tumor blocks. This is because the cores with a lack of expression were distributed throughout the tumor blocks, derived from various sections of the original tumor.

Another factor to consider when constructing TMAs for biomarker expression studies are both the characteristics of the specific biomarker as well as the expression pattern. For example, investigations of proteins involved in angiogenesis are particularly sensitive to ischemia during sample collection, which could greatly affect protein pattern (54). In contrast, vimentin, a ubiquitous antigen, is usually used as a reporter molecule to assess fixation and processing of tissue. It has been shown that vimentin is particularly sensitive to overfixation, and may be destroyed by this process (44). Therefore, the vimentin monoclonal antibody, V9, is used to assess the degree of formalin fixation. The MMR antibodies used in the current investigations are known to be relatively stable, based on their reliability in accurately identifying colorectal tumors with loss of MMR protein expression in which germ line mutations have subsequently been identified (32, 38, 55-57). Ultimately, when contemplating the use of a particular biomarker, systematic analyses should be done to determine the level of expression and the degree of uniformity required to accurately describe the outcome of interest (6, 50).

There were several strengths in the current investigation, including the prior quality work completed to validate the correlation of immunohistochemical protein

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**Figure 2.** Comparison of initial and follow-up results from MMR protein expression analyses: box plots of the results of staining from each of the three proteins (i.e., MLH1, MSH2, and MSH6) is shown. Initial block, the block initially analyzed; blocks 1 to 5, blocks used during the follow-up analyses. Specifically, the number of follow-up blocks were 1 (17 cases), 2 (14 cases), 3 (13 cases), 4 (10 cases), and 7 (5 cases). For comparisons of the initial block with each follow-up block, statistically significant findings are indicated (*, \(P < 0.05\); **, \(P < 0.001\)).
expression results between TMAs and full sections. In addition, we applied rigorous statistical methods to evaluate differences between central and peripheral punches. Despite these strengths, there remain a few limitations, including the relatively small sample size, sources of variability inherent in the multistep process of immunohistochemical analysis of archived tumor tissue, as well as tumor heterogeneity. Additionally, although it would have been preferable to normalize the staining intensity in cancer cells to adjacent normal cells, this was not feasible given the study resources, thus more research is needed to evaluate whether or not this enhances the ability to use suboptimally fixed tumor specimens for immunohistochemical analysis.

Findings from this study suggest that preferentially sampling from the periphery of archival tumor blocks when constructing TMAs in preparation for immunohistochemistry may be important because exposure to tissue fixatives is optimal in this location. This may reduce the likelihood of tissue fixation as a contributor to the lack of protein expression, and improve the reliability of the staining interpretation and overall validity of study results. Alternatively, another option is to stain full section slides from tumor blocks selected for TMA construction with vimentin, and only proceed accordingly. Ultimately, the introduction of the TMA enhances the ability to conduct biomarker research on a large number of tumor specimens in a cost-effective and efficient manner under uniform experimental conditions; however, data from the current investigation underscores the notion that there are several issues that should be considered when contemplating clinical research using TMAs for protein expression analysis of archival tumor tissue. Furthermore, it is critical to perform additional research to develop uniform processing procedures interpretation, including the development of optimal tissue fixation guidelines (i.e., thickness of sections, time to fixation, type of fixative used), with subsequent implementation. These standards have recently been implemented for processing of breast cancer samples (50, 58), and evaluating the implementation of these standards for all tumor processing should be considered.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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