Sampling Strategies for Tissue Microarrays to Evaluate Biomarkers in Ovarian Cancer

Jenny Permuth-Wey,[1] David Boulware,[1] Nikola Valkov,[2] Sandra Livingston,[2] Santo Nicosia,[2] Ji-Hyun Lee,[1] Rebecca Sutphen,[1] Joellen Schildkraut,[3] Steven Narod,[4] Alex Parker,[5] Domenico Coppola,[1] Thomas Sellers,[1] and Tuya Pal[1]

[1]H. Lee Moffitt Cancer Center, [2]University of South Florida, Tampa, Florida; [3]Duke University, Durham, North Carolina; [4]Centre for Research in Women's Health, Women's College Hospital Toronto, Canada; and [5]Mayo Clinic College of Medicine, Jacksonville, Florida

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)

Introduction

The development of tissue microarrays (TMA) for high-throughput molecular profiling of tumor specimens (1) has led to a rapid, relatively inexpensive, and efficient technique to analyze biomarkers in archived tumor specimens from large population studies. In contrast, conventional methods for the molecular analysis of tumor specimens, which typically require full section slides to be created from an original donor block (2), are labor-intensive and time-consuming. TMAs are constructed by obtaining small core biopsies from morphologically representative areas of paraffin-embedded tumor tissues and subsequently assembling the cores on a recipient paraffin block (3). A primary advantage of TMAs is that by their design, they allow for simultaneous analysis of specimens from a large number of cases on one slide, thus enabling all specimens to be exposed to uniform experimental conditions. Additionally, because cores rather than full sections are used, there is minimal destruction to the original donor material (1, 3). These notable benefits notwithstanding, a major challenge when using TMAs is to achieve accurate representation of the parent tumor tissues from which they were derived (4-6). Indeed, construction of TMAs must be completed under the guidance of a skilled pathologist in order to ensure accurate estimates of the prevalence and/or level of expression of the biomarker under investigation. Although investigators comparing molecular expression data between TMA cores and full sections have reported high rates of concordance (4, 7-12), factors that affect accurate representation include tissue heterogeneity, which varies according to tumor type, as well as tissue fixation. Epithelial ovarian cancers (EOC) are a particularly heterogeneous type of tumor (13-16), in part due to their large size. Furthermore, as with other tumor types, there is biological variability within and between individual EOC tumor blocks (17).

Another source of variability in biomarker studies is the use of immunohistochemical analysis, which provides a means to measure protein expression in various tumors. Although immunohistochemistry is widely used in surgical pathology as a diagnostic, prognostic, and therapeutic tool, there is variable consistency and poor reproducibility of results (2, 18-21). Published studies comparing immunohistochemical results from core-section–derived versus full-section–derived methods when using EOC specimens are lacking.

Received 7/31/08; revised 10/4/08; accepted 10/10/08.

Grant support: National Cancer Institute grants no. R01CA111914 (T. Pal) and no. K07CA108987 (T. Pal).

Requests for reprints: Tuya Pal, Department of Cancer Epidemiology and Genetics, Moffitt Cancer Center and Departments of Oncologic Sciences and Pediatrics, University of South Florida, MRC-CANCONT, 12902 Magnolia Drive, Tampa, FL 33612. Phone: 813-745-6239; Fax: 813-745-6525. E-mail: tuya.pal@moffitt.org

doi:10.1158/1055-9965.EPI-08-0713

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 EOC tumor blocks were obtained on 85% of the study sample.

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 model ATA-100; Chemicon International) as previously described (3). For each case, three replicate 1-mm cores were sampled from the center of 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.

The distribution of histologic subtypes of EOC was representative of the general population. Sample tracking was based on coordinate positions for each tissue core in the TMA recipient block; 4 μm sections were transferred onto separate TMA slides for immunohistochemical staining of each of the three MMR proteins under investigation (hMLH1, hMSH2, and hMSH6).

Immunohistochemical Staining for MMR Proteins. Deparaffinized, formalin-fixed paraffin-embedded tissues were microwaved in 1× EDTA (*hMSH2*; Chemicon) or Borg 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 was done by creating a new TMA comprised of representative cores obtained from the periphery of up to five 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 1. Histopathologic characteristics of EOC cases

	Total (n = 59)	Initial loss of expression (n = 17)	No initial loss of expression (n = 42)
	No. of cases (%)	No. of cases (%)	No. of cases (%)
Histologic subtype			
Serous	26 (44.0)	5 (29)	21 (50)
Endometrioid	11 (18.6)	5 (29)	6 (14)
Mucinous	8 (13.6)	3 (18)	5 (12)
Mixed cell, NOS	8 (13.6)	3 (18)	5 (12)
Adenocarcinoma, NOS	2 (3.4)	1 (6)	1 (2)
Clear cell	1 (1.7)	0	1 (2)
Other	3 (5.1)	0	3 (7)
Stage			
I	16 (27.1)	5 (29)	11 (26)
II	5 (8.5)	2 (12)	3 (7)
III	33 (55.9)	10 (59)	23 (55)
IV	5 (8.5)	0	5 (12)
Grade			
1	17 (28.8)	3 (18)	14 (33)
2	14 (23.7)	3 (18)	11 (26)
3	26 (44.1)	9 (52)	17 (40)
NA	2 (3.4)	2 (12)	0

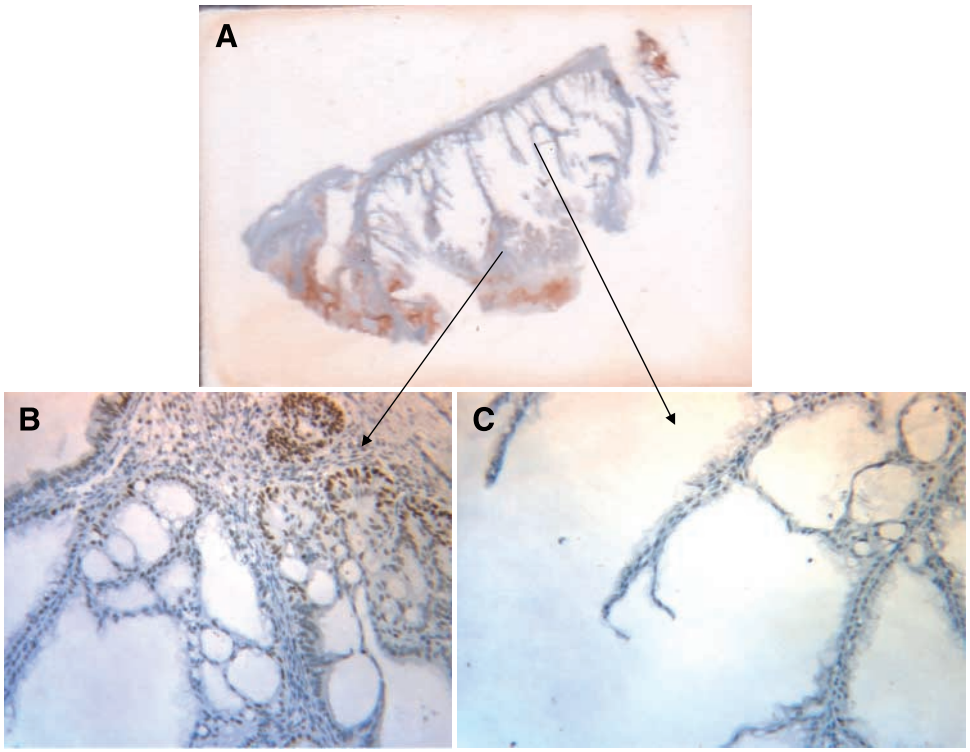

Figure 1. A. Full section slide of a mucinous adenocarcinoma stained for *hMLH1*, demonstrating lack of protein expression centrally and presence of expression peripherally (magnification, ×100). **B.** Centrally sampled core demonstrating lack of *hMLH1* expression (magnification, ×200). **C.** Peripherally sampled core demonstrating positive *hMLH1* expression (magnification, ×200).

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 $P \leq 0.001$) and MSH2 (all $P \leq 0.001$) 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).

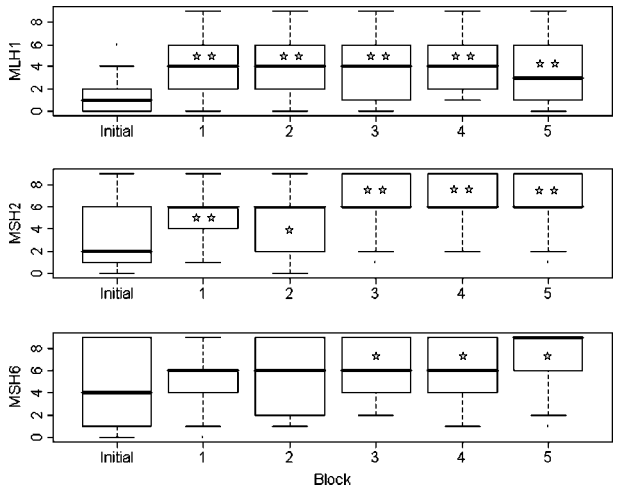

Figure 2. Comparison of initial and follow-up results from MMR protein expression analyses: box plots of the results of staining from each of the three proteins (i.e., MLH1, MSH2, and MSH6) is shown. *Initial block,* the block initially analyzed; *blocks 1* to *5,* blocks used during the follow-up analyses. 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 (*, $P < 0.05$; **, $P < 0.001$).

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 in 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

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.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–7.
2. Cregger M, Berger AJ, Rimm DL. Immunohistochemistry and quantitative analysis of protein expression. Arch Pathol Lab Med 2006;130:1026–30.
3. Fedor HL, De Marzo AM. Practical methods for tissue microarray construction. Methods Mol Med 2005;103:89–101.
4. Bentzen SM, Buffa FM, Wilson GD. Multiple biomarker tissue microarrays: bioinformatics and practical approaches. Cancer Metastasis Rev 2008;27:481–94.
5. Kallioniemi OP, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. Hum Mol Genet 2001;10:657–62.
6. Wang H, Wang H, Zhang W, Fuller GN. Tissue microarrays: applications in neuropathology research, diagnosis, and education. Brain Pathol 2002;12:95–107.
7. Hendriks Y, Franken P, Dierssen JW, et al. Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors. Am J Pathol 2003;162:469–77.
8. Hoos A, Cordon-Cardo C. Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations. Lab Invest 2001;81:1331–8.
9. Jourdan F, Sebbagh N, Comperat E, et al. Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression. Virchows Arch 2003;443:115–21.
10. Griffin MC, Robinson RA, Trask DK. Validation of tissue microarrays using p53 immunohistochemical studies of squamous cell carcinoma of the larynx. Mod Pathol 2003;16:1181–8.
11. Rubin MA, Dunn R, Strawderman M, Pienta KJ. Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol 2002;26:312–9.
12. Zu Y, Steinberg SM, Campo E, et al. Validation of tissue microarray immunohistochemistry staining and interpretation in diffuse large B-cell lymphoma. Leuk Lymphoma 2005;46:693–701.
13. Chiaffarino F, Parazzini F, Bosetti C, et al. Risk factors for ovarian cancer histotypes. Eur J Cancer 2007;43:1208–13.
14. Eltabbakh GH, Natarajan N, Piver MS, Mettlin CJ. Epidemiologic differences between women with borderline ovarian tumors and women with epithelial ovarian cancer. Gynecol Oncol 1999;74:103–7.
15. Kurian AW, Balise RR, McGuire V, Whittemore AS. Histologic types of epithelial ovarian cancer: have they different risk factors? Gynecol Oncol 2005;96:520–30.
16. Risch HA. Estrogen replacement therapy and risk of epithelial ovarian cancer. Gynecol Oncol 1996;63:254–7.
17. Scott M, McCluggage WG. Current concepts in ovarian epithelial tumorigenesis: correlation between morphological and molecular data. Histol Histopathol 2006;21:81–92.
18. Goldstein NS, Bosler D. An approach to interpreting immunohistochemical stains of adenocarcinoma in small needle core biopsy specimens: the impact of limited specimen size. Am J Clin Pathol 2007;127:273–81.
19. O'Leary TJ. Standardization in immunohistochemistry. Appl Immunohistochem Mol Morphol 2001;9:3–8.
20. Seidal T, Balaton AJ, Battifora H. Interpretation and quantification of immunostains. Am J Surg Pathol 2001;25:1204–7.
21. Wick MR, Mills SE. Consensual interpretive guidelines for diagnostic immunohistochemistry. Am J Surg Pathol 2001;25:1208–10.
22. American Cancer Society I. Cancer facts & figures 2007. [Internet] 2007 [cited 2007]. Available from: http://www.cancer.org/downloads/STT/CAFF2007PWSecured.pdf.
23. Watson P, Lynch HT. Cancer risk in mismatch repair gene mutation carriers. Fam Cancer 2001;1:57–60.
24. Vasen HF, Wijnen JT, Menko FH, et al. Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. Gastroenterology 1996;110:1020–7.
25. Aarnio M, Mecklin JP, Aaltonen LA, Nystrom-Lahti M, Jarvinen HJ. Life-time risk of different cancers in hereditary non-polyposis colorectal cancer (HNPCC) syndrome. Int J Cancer 1995;64:430–3.
26. Watson P, Lynch HT. Extracolonic cancer in hereditary nonpolyposis colorectal cancer. Cancer 1993;71:677–85.
27. Brown GJ, St John DJ, Macrae FA, Aittomaki K. Cancer risk in young women at risk of hereditary nonpolyposis colorectal cancer: implications for gynecologic surveillance. Gynecol Oncol 2001;80:346–9.
28. Aarnio M, Sankila R, Pukkala E, et al. Cancer risk in mutation carriers of DNA-mismatch-repair genes. Int J Cancer 1999;81:214–8.
29. Cederquist K, Emanuelsson M, Wiklund F, Golovleva I, Palmqvist R, Gronberg H. Two Swedish founder MSH6 mutations, one nonsense and one missense, conferring high cumulative risk of Lynch syndrome. Clin Genet 2005;68:533–41.
30. Arzimanoglou, II, Lallas T, Osborne M, Barber H, Gilbert F. Microsatellite instability differences between familial and sporadic ovarian cancers. Carcinogenesis 1996;17:1799–804.
31. Marra G, Boland CR. Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. J Natl Cancer Inst 1995;87:1114–25.
32. Marcus VA, Madlensky L, Gryfe R, et al. Immunohistochemistry for hMLH1 and hMSH2: a practical test for DNA mismatch repair-deficient tumors. Am J Surg Pathol 1999;23:1248–55.
33. Rigau V, Sebbagh N, Olschwang S, et al. Microsatellite instability in

colorectal carcinoma. The comparison of immunohistochemistry and molecular biology suggests a role for hMSH6 [correction of hMLH6] immunostaining. Arch Pathol Lab Med 2003;127:694–700.
34. Domanska K, Malander S, Masback A, Nilbert M. Ovarian cancer at young age: the contribution of mismatch-repair defects in a population-based series of epithelial ovarian cancer before age 40. Int J Gynecol Cancer 2007;17:789–93.
35. Geisler JP, Goodheart MJ, Sood AK, Holmes RJ, Hatterman-Zogg MA, Buller RE. Mismatch repair gene expression defects contribute to microsatellite instability in ovarian carcinoma. Cancer 2003;98: 2199–206.
36. Malander S, Rambech E, Kristoffersson U, et al. The contribution of the hereditary nonpolyposis colorectal cancer syndrome to the development of ovarian cancer. Gynecol Oncol 2006;101:238–43.
37. Rosen DG, Cai KQ, Luthra R, Liu J. Immunohistochemical staining of hMLH1 and hMSH2 reflects microsatellite instability status in ovarian carcinoma. Mod Pathol 2006;19:1414–20.
38. Chiaravalli AM, Furlan D, Facco C, et al. Immunohistochemical pattern of hMSH2/hMLH1 in familial and sporadic colorectal, gastric, endometrial and ovarian carcinomas with instability in microsatellite sequences. Virchows Arch 2001;438:39–48.
39. Singer G, Kallinowski T, Hartmann A, et al. Different types of microsatellite instability in ovarian carcinoma. Int J Cancer 2004;112: 643–6.
40. Pal T, Permuth-Wey J, Betts JA, et al. BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. Cancer 2005;104:2807–16.
41. Breslow NE, Clayton DG. Approximate inference in generalized linear mixed models. J Am Stat Assoc 1993;88:9–25.
42. Wolfinger R, O'Connell M. Generalized linear mixed models: a pseudo-likelihood approach. J Stat Comput Simul 1993;4:233–43.
43. Kenward MG, Roger JH. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 1997;53:983–97.
44. Battifora H. Assessment of antigen damage in immunohistochemistry. The vimentin internal control. Am J Clin Pathol 1991;96:669–71.
45. De Marzo AM, Fedor HH, Gage WR, Rubin MA. Inadequate formalin fixation decreases reliability of p27 immunohistochemical staining: probing optimal fixation time using high-density tissue microarrays. Hum Pathol 2002;33:756–60.
46. Werner M, Chott A, Fabiano A, Battifora H. Effect of formalin tissue fixation and processing on immunohistochemistry. Am J Surg Pathol 2000;24:1016–9.
47. Leong AS. Quantitation in immunohistology: fact or fiction? A discussion of variables that influence results. Appl Immunohistochem Mol Morphol 2004;12:1–7.
48. Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. Histopathology 2006;49:411–24.
49. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000;80:1943–9.
50. Moeder CB, Giltnane JM, Harigopal M, et al. Quantitative justification of the change from 10% to 30% for human epidermal growth factor receptor 2 scoring in the American Society of Clinical Oncology/College of American Pathologists guidelines: tumor heterogeneity in breast cancer and its implications for tissue microarray based assessment of outcome. J Clin Oncol 2007;25: 5418–25.
51. Ashworth A, Balkwill F, Bast RC, et al. Opportunities and challenges in ovarian cancer research, a perspective from the 11th Ovarian cancer action/HHMT Forum, Lake Como, March 2007. Gynecol Oncol 2008;108:652–7.
52. Naora H. The heterogeneity of epithelial ovarian cancers: reconciling old and new paradigms. Expert Rev Mol Med 2007;9:1–12.
53. Khalique L, Ayhan A, Weale ME, Jacobs IJ, Ramus SJ, Gayther SA. Genetic intra-tumour heterogeneity in epithelial ovarian cancer and its implications for molecular diagnosis of tumours. J Pathol 2007; 211:286–95.
54. Atkin GK, Daley FM, Bourne S, Glynne-Jones R, Northover JM, Wilson GD. The impact of surgically induced ischaemia on protein levels in patients undergoing rectal cancer surgery. Br J Cancer 2006; 95:928–33.
55. Bonis PA, Trikalinos TA, Chung M, et al. Hereditary nonpolyposis colorectal cancer: diagnostic strategies and their implications. Evid Rep Technol Assess (Full Rep) 2007;1–180.
56. Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Ruschoff J. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res 1997;57:4749–56.
57. Thibodeau SN, French AJ, Roche PC, et al. Altered expression of hMSH2 and hMLH1 in tumors with microsatellite instability and genetic alterations in mismatch repair genes. Cancer Res 1996;56: 4836–40.
58. Ross JS, Symmans WF, Pusztai L, Hortobagyi GN. Standardizing slide-based assays in breast cancer: hormone receptors, HER2, and sentinel lymph nodes. Clin Cancer Res 2007;13:2831–5.

Sampling Strategies for Tissue Microarrays to Evaluate Biomarkers in Ovarian Cancer

Jenny Permuth-Wey, David Boulware, Nikola Valkov, et al.

Cancer Epidemiol Biomarkers Prev 2009;18:28-34.