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Tissue Microarrays as a Tool in the Discovery and Validation of Predictive Biomarkers

Stephen M. Hewitt

Tissue Array Research Program/Laboratory of Pathology, National Institutes of Health/National Cancer Institute, Bethesda, USA.

Abstract

The tissue microarray (TMA) is the embodiment of high-throughput pathology. The platform combines tens to hundreds of tissue samples on a single microscope slide for interrogation with routine molecular pathology tools. TMAs have enabled the rapid and cost-effective screening of biomarkers for diagnostic, prognostic, and predictive utility. Most commonly applied to the field of oncology, the TMA has accelerated the development of new biomarkers, and is emerging as an essential tool in the discovery and validation of tissue biomarkers for use in personalized medicine. This chapter provides an overview of TMA technology and highlights the advantages of using TMAs as tools toward rapid introduction of new biomarkers for clinical use.

Keywords

Biomarker; FFPE; Immunohistochemistry; Pathology; Protein; RNA; Tissue microarray

1. Introduction

The concept of a multisample platform for pathology traces its origins to Hector Battifore, who developed approaches to present multiple samples of tissue on a single section (1). His methods were laborious and low throughput in nature, limiting the utility to primarily

genejock@helix.nih.gov.

4. Notes

1. Other fixatives, most notably Bouins (formaldehyde, picnc acid, and glacial acetic acid) and BS (formaldehyde and mercuric chloride), are suboptimal, as the tissue tends to be brittle and difficult to array.

2. For a detailed description of TMA production, see Hewitt et al. in Protein Microarrays: Methods and Protocols, 2004 (10).

3. The predominance ofIHC as an assay on TMA mirrors the use of whole tissue sections in general research. IHC is the application of an antibody to a tissue section to determine the histo- and cytologic localization of an antigen. Although generally not conceived as quantitative, the benefits of identifying the cell of interest and subcellular localization overcome the limitations of quantification. Performance of an IHC assay on TMAs is not different from IHC on whole tissue sections, but the approach has uncovered deficiencies that have been difficult to study previously (10, 14, 43).

4. Predictive biomarkers, especially those indexed on survival, may be assayed (or reassayed) at the conclusion of the trials. Additionally, it is routine to test patient specimens at relapse, not at diagnosis, for the use of predictive markers, not to mention the application of new markers to antecedent patients' samples as these assays are developed and approved. In so far as the *total test* paradigm, the tissue specimen has a set of specified preanalytic elements appropriate for the assay. Fixative type is essential, and fixation time is an element of specification for some assays. There is no doubt that alternative tissue processing methods alter the specimen and may render the specimen inappropriate.

5. Formalin is an aqueous form of formaldehyde, the simplest aldehyde, which acts to cross-link proteins, as well as nick and cross-link (to proteins most commonly) nucleic acids.

6. It is believed that this higher quality is a result of more complete tissue dehydration. Alternative tissue processor methodologies have not been rigorously validated (16).

control slides for the development of immunohistochemical assays. In 1998, Juha Kononnen (2), working in the National Human Genome Research Institute (National Institutes of Health), developed the first tissue microarray (TMA) and functional tissue arrayer, which allowed the production of high-throughput TMAs with a relatively simple instrument. A key feature of these arrays was the precise and orderly arrangement of the tissue cores in the recipient block so that the individual tissue cores could be traced back to the individual patients and their clinical information (3). Although primarily conceived as a research tool for understanding protein expression, it was not long before the TMA was being used to confirm the nature of prognostic and predictive biomarkers (4, 5).

The most common assay used in conjunction with TMAs is immunohistochemistry (IHC). This combination provides a widely used tool for the rapid translation of biomarkers into clinical benefit. TMAs are constructed of archival tissue samples obtained as a part of treatment and diagnosis. IHC applied to TMAs is now a common routine method for validation of gene and protein array data (1, 2, 5), image analysis technology development (6), and molecular research (4). By utilizing antibodies, a common tool of biomarker discovery and research, it requires only a fraction of the effort to move from the research setting to evaluate hundreds of tissue samples at one time with an already clinically accepted assay. The remaining challenge is collection and annotation of the samples (7). Fundamentally, the TMA shifts the balance of how tissue is interpreted with reference to defining disease. Previously, histomorphologic features of a tumor have been used to characterize the behavior of a tumor. Increasingly, it is the expression of specific biomarkers, as detected by IHC, which provides molecular predictors of tumor behavior.

2. Materials

2.1. Samples

1. Within the field of cancer research, TMAs have focused on malignant lesions or screening cohorts for examining normal and dysplastic lesions, although any tissue type can be used to construct TMAs (8, 9).
2. Translational research is founded on principles of *translating* findings from molecular biology to clinical utility; therefore, use of clinically derived tissue, handled according to the same precepts of surgical pathology, is preferred (7).
3. Formalin-fixed, paraffin-embedded tissue is the most commonly applied tissue for TMA construction, but this is a reflection of its predominance in pathology archives and practice (10). Alternative fixatives can be utilized; however, knowledge of the fixative composition is essential, as assays are developed based on these preanalytical features. Ethanol-fixed tissue is also encountered in research settings (9, 11) (see Note 1).

2.2. Tissue Microarray Construction

1. A histopathologist, via microscopic examination, identifies the regions of interest on corresponding tissue section slides.

2. Areas corresponding to these regions of interest are cored from the original donor blocks. A TMA block consists of a recipient block of paraffin into which cores of tissue have been placed with needles after extraction from paraffin donor blocks.

3. A number of instruments now exist for the construction of TMAs, offering a variety of diameters of tissue core size, typically from 0.6 mm (the field of view of a 40× objective on a standard pathology microscope) to 2 mm or greater (see Note 2).

4. Diameter of the tissue cores determines the density of the array. With 0.6-mm needles, arrays on the order of 500 cores are routine while 2-mm cores result in arrays of 40 or so samples.

5. The recipient (TMA) block is sectioned with a microtome onto rrucroscope slides, which are subsequently used for specific assays.

2.3. TMA Assays

1. The assays performed on TMAs are as diverse as the arrays pursued on all tissues. The most common assay is IHC, accounting for approximately 95% of all stains (10) (see Note 3).

2. The routine hematoxylin and eosin (H&E) stain accounts for ~3% of assays, and the last 1 % are in situ assays for either RNA or DNA. The H&E stain is a routine stain for diagnostic histoand cytomorphologic examination. As such, it is typically used on sections from every TMA block to confirm the diagnosis of the tissue present, usually on every 50th section.

3. Methods

3.1. General Process

3. 1. 1. Identification of Material—Identification of appropriate tissue material and patient cohorts for development of clinical predictive assays is essential. Key points that must be addressed include ethical approval and access to sufficient numbers of cases, which have sufficient diagnostic material for assay and reassay during the development process (10). Lastly, the material must be well-annotated both at the diagnostic and the clinical outcomes levels. Depending on the phase of predictive biomarker development, an epidemiologic approach must be employed to determine the impact of different populations and the molecular differences of the disease between populations. This process is typically the most time consuming, and the time and effort required are often underestimated.

3.1.2. Tissue Fixation and Embedding—The process of preparing formalin-fixed, paraffin-embedded tissue includes (1) acquisition of tissue from clinicians and surgeons; (2) preparation of the tissue specimen such that it is appropriate for fixation and processing (e.g., cutting/ dissecting the tissue into pieces approximately 10 × 10 × 4 mm); (3) fixation, most commonly in formalin; and (4) tissue processing, which is the process of replacing the aqueous environment of tissue with paraffin using a series of alcohol dehydration steps.

Ultimately, the paraffin-impregnated tissue is embedded in a paraffin block, and sectioned onto glass slides for staining. The block is archived, and may eventually be used in a TMA.

3.2. Tissue Microarray Construction

A number of excellent reviews and technical sources are available on the construction of TMAs (12, 13). In so far as construction and validation of TMAs for predictive biomarkers, there are two essential elements: redundant arrays and representativeness of the arrays against the anticipated clinical standard - analysis on whole sections (14). These issues are intertwined. Given the efforts of obtaining a trial or cohort for analysis for a predictive marker, it would be foolish to produce only a single array. Replicate arrays are invaluable for assay development, reproducibility assays, and comparison of new reagents and methodologies, let alone comparison of other biomarkers. The number of replicates that can be produced is a function of the tumor type, size of specimens available for analysis, and array design. Simply, “more is better” to ensure a larger sample size for adequate tumor/stroma area and multiple pieces of tissue from the same or similar blocks. The issue of tissue core size can be a function of preference and number of samples. Although significantly more samples can be applied to a section with 0.6-mm needles, 1.0-mm needles may be chosen for a number of reasons. Larger cores should not be assumed as a simple solution to the issue of representativeness (14), and may reduce the number of replicates; however, in some instances, such as biopsies, they are a preferred solution (9).

Representativeness of a TMA is very challenging and the literature is confusing at best. The goal is to ensure that a TMA accurately represents what would be seen on whole tissue sections. In the context of a predictive assay, this is demonstration that the results of the assay on a TMA would give the same prediction of response or outcome if applied to whole sections cut from the same cases (14). A strict definition is that the TMA demonstrates exactly the same biomarker profile as determined by examination of the whole sections, not just a statically equally proportion. Fundamentally, the only means to accomplish this is to test this comparison, based on the proven utility - a catch-22. It is inappropriate to assume that the representativeness of one marker predicts the representativeness of the next marker. They are independent events. Ultimately, the only solution is a best effort and, then when a potential marker has been found, validation on whole sections (10). This is where replicate arrays are important, as staining of replicate arrays allows determination of how many cores are required to approximate a whole section. Many investigators perform pilot tests on core size and replicate numbers to solve these problems before they construct a large TMA.

3.3. Input Sample Quality

It cannot be emphasized enough that tissue quality is a first-order concern (15). Despite the widely used phrase “per standard protocol,” there are functionally no standard protocols between hospitals, and differences between countries of origin are significant (16). To date, no factor in specimen handling has been identified that does not impact quality when measured by the most stringent assays. However, useful tissue biomarkers do exist, demonstrating the intersection of robust biomarker assays and quality tissue preparation. Two factors most commonly associated with specimen preanalytical variability are tissue collection and tissue preparation.

3.3. 1. Tissue Collection and Warm Ischemia Time—Acquisition of tissue from the surgeon and clinician, before fixation, is referred to as the warm ischemia time, during which time the tissue is devitalized but not preserved. Prolonged warm ischemia times are clearly associated with suboptimal specimens, which demonstrate altered gene and protein profiles (17–20). However, it is impractical to collect many tissues with extremely short warm ischemia times, making many markers of tissue hypoxia impractical for clinical implementation. Reasonable goals for warm ischemia time are under 30 min if one wishes to maintain the fidelity of the in vivo state of the nucleic acids and proteins (18, 19).

3.3.2. Tissue Preparation—Preparation of the tissue in the pathology laboratory is called “grossing” and includes the process of inking, dissecting, and sectioning of the specimen (16). Depending on the specimen, it may be submitted in toto, in total without the aforementioned steps, such as a small biopsy, but excisional biopsies and expatriations of masses or organs require extensive grossing. Depending on specimen type and size, all of the tissue or a small sampling of the tissue is selected for tissue processing and microscopic examination. The key issue is that this is done in a timely manner and the sections are of appropriate size for tissue processing.

3.4. Biomarker Validation

When an assay developer constructs an IHC assay and correlates it with some utility, the process is called *validation*. When an end user purchases and applies the assay, the user goes through a process of *verification*, in which the end user demonstrates the assay performance as intended (21–24). In so far as development of a validated assay, it is essential to test as many potential variables as possible to ensure that the assay is accurate - reporting a true result. For material obtained from a single center, this is often straightforward; however, in multi-institutional trials, the effects of preanalytic variables, typically fixation and processing, must be incorporated into the validation strategy. In a very simple fashion, this can be determination that the distribution of results from the assay is the same between sites and that any variance in this distribution can be accounted by other methods.

3.5. Proficiency Testing

Another simple element of validation is appropriate crossover testing, also known as proficiency testing, With TMAs, this approach is far simpler than that was previously possible (22, 24). In a crossover validation, TMAs containing tissue of the same diagnostic specifications are shared between two laboratories, which perform the same assay on the specimens. When an assay is robust, the results in lab A on TMA A are identical to the results in lab A on TMA B while at the same time lab B gets identical results on TMAs A and B.

3.6. Total Test Approach to As

One element of the development of a predictive biomarker is the concept of the *total test assay*. An assay is calibrated to give a result and this calibration is dependent on the elements of the assay - specimen, analyte, as well as other reagents, and interpretation. For many biomarkers, especially those used in diagnosis (often referred to as markers of lineage), calibration of the assay is simple as the results are typically binary - presence or

absence of the marker. In contrast, predictive biomarkers are often based on a specific cutoff or predetermined level of the biomarker of interest. As a result, the calibration of the assay is more specific. To obtain this specificity of the assay, the parameters of the assay require stringent specification.

To determine the predictive value of a biomarker, the assay is defined and performed on a series of specimens for which the response to therapy is determined (25). From this test, a correlation of the assay result with response is developed. Previously, a biomarker was used for selection of patients, and only those patients with a positive result were treated. Although this approach does work, it fails to generate a positive and negative predictive values of the biomarker as it relates to response, as patients who were biomarker negative were never challenged with the treatment, and their correlation with outcome was not determined (26).

The correlation of the biomarker with response is that of a *total test*. Alterations in the assay directly result in alterations in correlation with response. Most commonly, the alteration is introduction of new/alternative antibodies or detection systems, with the belief that these new reagents, typically with higher molecular specificities, result in a superior assay. It is inappropriate to make these assumptions, and the correlation requires formal testing. Another common failure is specification of the specimen, where specimens not handled as specified in the original assay are tested and give inappropriate results. This is an all too common problem, and stems from both poor specimen preparation as well as inadequate specification of the assay (27).

3.7. Interpretation

For over a century, pathology has been defined by interpretation of histomorphology and cytomorphology, generating a description, leading, within the context of the patient, to a diagnosis. This process was mediated by the gross examination of tissue and microscopic examination of slides stained with compounds of differing specificities to different classes of biomolecules.

A paradigm shift occurred with the advent of IHC in which an antibody is utilized to identify a specific protein (or other antigen) in tissue. Interpretation of IHC requires a description of where the antibody-antigen complex is detected- both at the histomorphologic and cytomorphologic levels, combined with qualification of its abundance.

With this molecular approach, the definition of disease began to shift from pattern and appearance of cells to definition by the presence of individual proteins that define the disease. Cellular pattern and appearance continue to be the lynch pin of diagnostic pathology while the use of IHC is an adjunct used for confirmation of the diagnosis, along with the clinical features of the patient. As the breadth of antibodies widens, a panel of antibodies may be more prognostic than histomorphologic-based grading, not to mention the utility of predictive biomarkers that may be applicable to individualized therapy.

In practice, the majority of immunohistochemical stains are interpreted based on the anticipated localization of the antibody-antigen complex (nuclear, cytoplasmic, membranous, or a combination thereof) in a binary (present or absent) fashion. In general,

the binary interpretation is based on the staining of *any* of the appropriate cell types and subcellular compartments. Unfortunately, the basis on which these relationships are founded are too frequently made on small case reports of selected tumors and never tested appropriately to define both false-positive and false-negative rates. Many markers probably lack the specificity ascribed to them (28).

3.7. 1. Prognostic Marker—The application ofIHC to prognostic markers requires establishing a "cutofP' value that is applied to the interpretation to develop a prognostic relationship. Common cutoff values are 1 and 10%, meaning that at a minimum this predefined number of cells of interest should express the marker of study to constitute a positive reaction and a clinically relevant prognostic correlation. In this context, the application of a total test is especially important. Should the assay, specimen, or interpretation of the assay be altered, the correlation of immunohistochemical reaction with a prognosis is lost.

3.7.2. Predictive Biomarkers—Predictive biomarkers typically adhere to the same principles as prognostic biomarkers. In fact, most predictive biomarkers are prognostic as well. In the same way, a graded approach to the result of an immunohistochemical assay would theoretically result in a probability of response to an intervention or drug. If a sufficiently large cohort of patients is both tested for a predictive biomarker and are treated with the agent/intervention to which the biomarker predicts response, it would be possible to define, for every inter-pretative step in the immunohistochemical assay, a probability of response to the agent/intervention. In practice, this is not done; rather a cutoff is set and patients above the cutoff are considered positive and treated. This approach results in potential undertreatment of patients who have a lower but definable probability of response while those who are positive have a probability of not responding. In practice, this is one rationale for treating a woman who has any estrogen receptor-positive breast cancer tumor cells (29). The probability of response is sufficiently high compared to the risk and cost of the therapy to warrant treatment.

It should be noted that theory is theory, and not practice. To determine these relationships, three elements are essential: (1) a total test assay, incorporating interpretation; (2) an interpretative schema that allows multiple categories in a fixed progression that correlates with increased expression; (3) a clinical trial design that treats patients who are biomarker positive and negative and is of sufficient size to meet the power calculations of the assay (26, 30). Within the realm of trials, it is essential to demonstrate that the effect of the biomarker is selection of the patients, with appropriate change in outcome, and not the underlying prognostic impact of the biomarker (25, 30).

3.8. Image Analysis

To answer the complexity of immunohistochemical interpretation, image analysis/image quantification has been developed as a means of standardization and quantification of IHC. It must be emphasized that image analysis is but the last step of the process of a test, and that if the test is not appropriately specified, calibrated, and carried out image analysis cannot improve results. Tools are available to define the cell type of interest (who and where);

however, none of these tools are foolproof and always require human verification/ supervision. Image analysis is clearly superior at the quantification elements ofIH C-how much and how many. By generating continuous data, more sophisticated analysis is feasible, especially for prognostic and predictive biomarkers (6). Incorporating image analysis as an element of interpretation is a crucial facet of personalized medicine.

As these issues apply to TMAs, it is the development of the TMA that has driven the development of these assays (31). Although prognostic and predictive biomarkers were previously identified, the advent of the TMA has opened up the pipeline to rapid testing of potential biomarkers. alpha-Methylacyl coenzyme A racemase (32) is an excellent example of a biomarker identified by these means. It is anticipated that TMAs will likewise leverage image analysis to result in a predictive assay that cannot be accurately reproduced by manual interpretation, but is robust with a well-designed image analysis algorithm, as exemplified in the utility of survivin as a prognostic biomarker in breast cancer (31).

3.9. Multiplexed Markers

Despite the obvious high-throughput nature of TMAs, in general, there is a lack of publications that evaluate biomarkers as panels for personalized medicine. The majority of papers examine a single protein or examine multiple proteins individually. Rare papers have examined panels of biomarkers; however, they have failed to define the utility of the individual biomarkers in a panel and by their nature are difficult to reduce to clinical utility (33). Shau et al. demonstrate the potential to utilize two markers concurrently, although only within a complex model of tumor progression and survival (9).

3.10. Discovery

Although TMAs are routinely used in testing and validation of biomarkers for personalized medicine, their application is not limited to human pathology samples taken directly from patients. The general platform of an array of cellular material can be applied to cell lines grown in vitro or xenograft samples. Cell line microarrays (CMAs) and xenograft microarrays (XMAs) are used at the discovery level as well as tools toward assay development (13).

In their role as discovery tools, CMAs and XMAs are potent tools for transition from experimental platforms, such as western blotting, to a clinically relevant immunohistochemical assay (13). Direct information regarding cell types affected by drugs can be derived, as well the capacity to interrogate pathways, to define the best biomarker for the intended therapy. Often, new agents target specific proteins or classes of proteins, and construction of directed assays toward these targets is useful prior to clinical trials to prove the mechanism of action. However, it should not be assumed that this approach would result in the most clinically relevant biomarker. In some instances, downstream markers may function as more robust markers of predictive medicine. Two primary factors are responsible for alternative (typically, downstream) targets providing a better correlation with response. One is the limitation of antibodies; some epitopes are appropriate for IHC in FFPE tissue, but differences in antibody affinities may render a particular epitope/protein as a suboptimal biomarker. In some instances, the solution may be to select a neighboring epitope within the

same antigen. For example, with HER2/neu, the therapeutic monoclonal antibody Trastuzumab (Herceptin®) binds the extracellular domain of HER2 while the antibodies used in the HercepTest® target the intracellular domain. The second issue is molecular cross talk. Alternative members of a signaling pathway may be better reporters of signaling due to differences in phosphorylation or magnitude of expression. Aspects of these relationships may be exhibited by agents targeting mTOR and AKT in the PTEN-PI3 Kinase pathway (34, 35).

3.11. New Methodologies

3. 11. 1. Multiplex Assays—Rimm et al. have developed a fluorescence-based, multiplex-based immunohistochemical assay approach (36) that has been very successful at identifying relationships of biomarkers with clinical outcomes that were not previously appreciated (37). Another approach is a multiplex immunoblot method, by which proteins are transferred from an FFPE tissue section to specially treated membranes which are then probed like an immunoblot (38). This method, a bit of a hybrid of western blot and IHC, provides histo-geographic spatial resolution. The primary advantages of this approach are multiplex (up to five antibodies), normalization against total protein content, and quantitative data, allowing development of ratio-based biomarkers. The data generated by this multiplex immunoblot technology has been replicated by IHC; however, the quantification and normalization methodologies appear to offer some benefit (34, 38, 39).

3.11.2. Fourier Transform Infrared Spectroscopy Coupled with TMA—In a more exotic application, Ira Levin's group has applied Fourier transform infrared spectroscopy (FTIR) to TMAs (40). In the first demonstration of the capacity of infrared spectroscopy of tissue, Fernandez et al. were able to generate a set of metrics that could segregate prostate tissue into its individual tissue components and with reasonable success identify cancer vs. benign prostatic epithelium (41). Without the high-throughput nature of TMAs, the baseline metric would not have been feasible. This approach is sufficiently robust such that the original metrics can be applied to other tissue types and sources (including frozen tissue) and function as classifiers.

3.11.3. CMA Coupled with Fourier Transform Infrared Spectroscopy—In an application more focused on personalized medicine, Chen et al. (42) utilized FTIR to define metrics of molecular responses to a drug. In their experiments, peripheral blood leukocytes (PBLs), arrayed in a CMA-like format, were exposed to histone deacetylase (HDAC) inhibitors in vitro, and a series of metrics that defined and quantified the changes in lysine acetylation were developed. The assay requires no specimen processing or affinity reagents, depending solely on changes in the vibrations of peptides based on the presence or absence of an acetyl group. In a Phase 0 clinical trial, patients were treated with HDAC inhibitors, PBLs were collected, and the alterations in acetylation were measured in response to drug treatment.

3.12. Troubleshooting: Tissue Preservation is the Major Source of Variability

Unfortunately, it is impossible at this time to provide strict guidance on the best practices, and the current effort is to educate the community on this issue as well as continue research

to better characterize these differences. Defining “best” is very challenging, as the factors impacting FFPE tissue quality are interlocking. The two salient issues are: (1) producing an archival-quality FFPE tissue and (2) treating these preanalytic variables as elements of the specification of the specimen within the concept of a total test (see Note 4).

Fixation is a key, and too often mismanaged step, in the tissue preservation chain. Although there are a number of historic and recently described fixatives, the number of tissue fixatives in daily use has diminished over the last decade.

1. Ten percent neutral buffered formalin is the overwhelming choice of fixative worldwide (16). Buffer formulations are varied and have some impact on both RNA and protein in tissue, although this remains poorly characterized (20) (see Note 5).
2. Adequate fixation is a function of time, but requires approximately 24 h, depending on specimen size (18). Despite the assumption that overfixation is worse than underfixation, data demonstrates that underfixation leads to significant quality problems and unreliable assays at both the RNA and protein levels (18, 20). Current recommendations vary greatly; however, 16–32 h are a relatively safe window, as well as obtainable in clinical practice.
3. Fixative volume should be a minimum of ten times the volume of the tissue.
4. Tissue processing protocols impact specimen quality and extent of cross-linking (16, 18, 19). Limited data supports that a slower process, with vacuum and heat, results in high-quality specimens (20) (see Note 6).

3.13. Summary

The TMA has become an essential platform for validation of predictive biomarkers. This platform brings high-throughput technologies together with routine clinical specimens to create validated assays. The TMA is frequently the platform in which a final clinical assay is developed, having been discovered via other methodologies, but in some instances TMA is the platform for discovery of novel predictive biomarkers.

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