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Practical aspects of planning, building, and interpreting tissue microarrays: The Cooperative Prostate Cancer Tissue Resource experience

A. Kajdacsy-Balla · J. M. Geynisman · V. Macias · S. Setty · N. M. Nanaji · J. J. Berman · K. Dobbin · J. Melamed · X. Kong · M. Bosland · J. Orenstein · J. Bayerl · M. J. Becich · R. Dhir · M. W. Datta · The Cooperative Prostate Cancer Tissue Resource

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Abstract This is a review of several new approaches developed at or adopted by the Cooperative Prostate Cancer Tissue Resource (CPCTR) to resolve issues involved in tissue microarray (TMA) construction and use. CPCTR developed the first needle biopsy TMA, allowing researchers to obtain 200 or more consecutive cancer sections from a single biopsy core. Using radiographs of original paraffin blocks to measure tissue thickness we developed a method to produce TMAs with a larger number of usable sections. The modular approach to plan TMA construction is also a novel concept wherein TMAs of different types, such as tumor grade TMAs, metastasis TMA and hormone refractory tumors TMA can be combined to form an ensemble of TMAs with expanded research utility, such as support for tumor progression studies. We also implemented an open access TMA Data Exchange Specification that allows TMA data to be organized in a self-describing XML document annotated with well-defined common data elements. It ensures inter-laboratory reproducibility because it offers information describing the preparation of TMA blocks and slides. There are many important aspects that may be missed by both beginners and experienced investigators in areas of TMA experimental design, human subjects protection, population sample size, selection of tumor areas to sample, strategies for saving tissues, choice of antibodies for immunohistochemistry, and TMA data management.

Keywords Tissue microarrays · Experimental design · Immunohistochemistry · Prostate cancer

A. Kajdacsy-Balla (✉) · J. M. Geynisman · V. Macias · S. Setty · N. M. Nanaji
Department of Pathology, University of Illinois Chicago, 1818 W. Polk St, Chicago, IL 60607-7053, USA
e-mail: aballa@uic.edu

J. J. Berman · K. Dobbin
National Cancer Institute, Bethesda, MD, USA

J. Melamed · X. Kong
Department of Pathology, New York University School of Medicine, New York, NY 20892-2590, USA

M. Bosland
Departments of Environmental Medicine and Urology, New York University School of Medicine, New York, NY 10016, USA

J. Orenstein · J. Bayerl
Department of Pathology, George Washington University, Washington, DC 20052, USA

M. J. Becich · R. Dhir
Department of Pathology, University of Pittsburgh Medical School, Pittsburgh, PA 15260, USA

M. W. Datta
Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

Introduction

Tissue Microarray (TMA) is a type of tissue assay that allows the researcher to simultaneously visualize and study tissues from several, even hundreds of patients. In this era of genomics and proteomics, TMAs have a distinct usefulness and interest. This review article details the procedural considerations of planning and building a TMA for both novice and seasoned researchers. While our experience in design and data is focused on prostate cancer tissues as part of the Cooperative Prostate Cancer Tissue Resource (CPCTR), these techniques are

applicable to any type of tissue and other diseases. CPCTR is a multi-institutional tissue bank sponsored by the National Institute of Cancer (Bethesda, MD, USA) with a large number of prostate cancer specimens (more than 6,000 subjects) with associated clinical and outcome data available for the research community (Melamed et al. 2004, www.prostatetissues.org). The purpose is to review aspects of TMA design, construction, and actual use that may be easy to miss.

Types of TMA design

Most, if not all TMAs will be used for more than one research project. The exact use of the first few projects may be known before the TMA is built, but a single TMA paraffin block could be used for a 100 different projects or more. Therefore, a considerable amount of time should be invested in planning in order to maximize resources and to address statistical issues. Ideally one would like to know ahead of time about the research interests of possible users of the end product. Several types of TMA have been used by CPCTR and other investigators for the study of cancer.

Outcomes-based TMA

Cases with the longest clinical follow up and best-documented outcome measures are the most useful in this type of array. They are mostly used for testing and validating candidate cancer prognostic markers. As discussed below, the main concern in building this type of TMA is to make sure the number of cases available for arraying has enough statistical power to address these issues. Outcomes TMAs usually include only tissue cores that contain tumor. Low percentage of cases that are lost to follow up is a pre-requisite for building a TMA based on consecutive cases for outcome studies. An innovative, alternative way of designing outcomes-based TMAs was used by CPCTR. Instead of using all consecutive cases, some with and some without complete follow up, we selected only cases with more than 5 years of post-prostatectomy clinical follow up, cases that had sufficiently large numbers of post-operative serial serum PSA measurements to be able to evaluate biochemical recurrence. In this type of TMA each case of the group of subjects that had PSA biochemical recurrence was matched to a non-recurrence case based on age, race, Gleason grade sum score, and pTNM stage (matched-pair case-control design), a method that decreases the number of cases arrayed without a significant decrease in the statistical power of the experiment.

Progression-based TMA

This type will have cores that represent the entire spectrum of disease, starting with tissues from normal individuals and ending with tissues obtained from distant metastases of patients who are refractory to treatment. Ideally a progression TMA will include cases without cancer (obtained from organ transplant donors whose families consented for inclusion in a rapid autopsy program), pre-malignant disorders, and histologically normal tissues from areas adjacent to a cancer, histologically normal tissues from areas distant to a known cancer, cancers at various stages and grades, as well as regional and distant metastases. A single subject may contribute with several of these tissue cores. Understanding the distinction between normal tissue obtained from an individual without known cancer and normal appearing tissue obtained from an individual with known cancer is crucial in the interpretation of results since there are always concerns about the phenomenon of ‘‘field effect’’ in the entire prostate of someone who has a localized prostate cancer (Chandran et al. 2005).

Tumor grade TMA

This is similar to progression-based TMAs. It is useful for evaluating the frequency of a marker throughout the spectrum of tumor differentiation. Because tumor grading is somewhat subjective, it is important to use set criteria and to enlist the help of more than one pathologist to grade the tumors, with a discussion to reach consensus on specimens that get discordant grading assignments (Oyama et al. 2005). CPCTR has an example of such type of TMA, the TMA2 or Gleason Grade TMA, www.prostetissues.org.

Tumor heterogeneity TMA

This design includes many cores from each subject in order to address heterogeneity of tissue marker expression in the same lesion. For example, a heterogeneity type TMA may include two core samples from the center of the tumor, two from each quadrant of the tumor, two from its advancing edge, and two from foci of metastasis.

Consecutive cases TMA

The most common design in the literature is the consecutive cases array where all the available cases are used. For example, the first TMA constructed by CPCTR (TMA 1) for public use has this type of design

(www.prostatetissues.org). This type of TMA is frequently used for studies where the goal is to evaluate the frequency of expression of a given antigen in cancers of a specific organ. Because consecutive cases TMAs include all cases available for arraying, it may be the best approach if one is building only one TMA. The major drawback for this approach is that many users will have to disregard a considerable number of the tissue cores in the TMA because these cores do not address the experimental question, obviously not a frugal use of the available tissue resources.

Specialty TMA

Sometimes a TMA that does not conform to the usual types of TMA is built to fit a particular research need. For example, at CPCTR we are now building three specialty TMAs: A TMA that has similar numbers of age, Gleason grade and stage-matched groups of African Americans and Caucasians; a TMA that has most of the cases with metastatic tissues from the CPCTR collection; and a collection of cases with both hormone refractory and hormone naïve tumors. These specialty TMAs have been named Ethnic TMA, Metastasis TMA, and Hormone Refractory TMA, respectively.

An alternative solution to the dilemma of what type of TMA to build is to use a modular approach, where TMAs of different types can be used to complement each other. For example, in order to substitute for a progression type TMA at CPCTR, the previously mentioned Metastasis TMA can be used to complement the TMA1 (a consecutive cases type TMA). To our knowledge we are the first group to use this approach for building a set of TMAs that maximizes the use of tissue resources. Because metastatic tissue is not readily available in the department of pathology archival materials, we try not to use metastatic samples in a TMA unless they are strictly necessary.

TMA from biopsy material

Most TMAs are based on large specimens such as whole prostates, breast cancer excisional surgeries, removal of segments of colon that contain cancer, etc. For some research purposes however, the use of needle biopsies is more appropriate and sometimes it is all that is available. At CPCTR we developed the first method to produce TMAs from needle biopsies (Datta et al. 2005). Thin needle prostate biopsy paraffin-embedded tissue cores are available from most departments of pathology as leftover after several cuts are obtained for diagnostic purposes. Because these specimens are so thin, only 4–10 residual slides can be cut from such material before depleting it. In summary, for this type of TMA, the leftover tumor tissue is carved out of the paraffin block with a razor blade, re-embedded in a ‘‘trough’’ made of aluminum foil that is filled with liquid formalin. After the paraffin becomes solid, the cancer tissue core looks like a very thin paraffin crayon pencil. The core is then manually placed vertically into one of previously cut holes in a recipient paraffin block. Up to 200 paraffin sections can be obtained from one of these TMAs.

Tissue culture cells array

Cells obtained from tissue cultures can be made available in a TMA format for various purposes. At CPCTR we use them for controls as one of the cores within tissue-based TMAs. One can also use cells before and after specific treatments, transfected cells, or simply as control TMA cores because these cell lines have been extensively used in the literature and much is known about their immunohistochemical profile (Moskaluk and Stoler 2003; Li et al. 2005; Montgomery et al. 2005).

Population sample size issues

A crucial step to the success of building a TMA is to choose an appropriate population size. To calculate the specific needs of the future studies, it is important to enlist the help of a biostatistician from the beginning. It also helps to use one of many available statistical software packages that can calculate statistical power such as StatMate (GraphPad Software, San Diego, CA, USA) and similar products. In Table 1 there are some practical examples of the number of subjects needed to obtain reliable statistical results. In one of the examples, we pulled 20 randomly picked sets of subjects from a population of 954 prostate cancer cases with known outcome (analyzable serial post-prostatectomy serum PSA curves in order to diagnose PSA biochemical recurrence versus non-recurrence; >5-year follow-up). In the entire 954 subjects population 437 had histological Gleason score of 6 and below, and 517 subjects had Gleason grade 7 and above. The recurrence rates in these two groups were 13 and 36%, respectively (2.8-fold increase). The Gleason score is one of the best prognostic indicators for prostate cancer. Using chi-square analysis, we determined the number of times that two well-known prognostic markers would have shown statistical significance. Even with this large difference in recurrence between

Table 1 Practical exercise of the number of subjects needed to obtain reliable statistical results

Sets	pT2 versus pT3 cases (significant χ^2/total sets)	Gleason score <6 vs. >7 (significant χ^2/total sets)
20 sets of 50 cases		09/20
20 sets of 100 cases		14/20
20 sets of 200 cases		19/20
20 sets of 300 cases	15/20	20/20
20 sets of 400 cases	19/20	20/20
20 sets of 500 cases	20/20	20/20
Entire set (954 subjects)	22.8% vs. 35.9% (1.57-fold)	13% vs. 36% (2.8-fold)

the two groups, 300 patient cases were needed in order to obtain significant results in all of the randomly picked 20 trial sets extracted from the whole population. When we used sets of only 100 patients, we found that Gleason grade grouping was statistically significant in only 14 of 20 random sets of subjects. By comparison, pathological tumor stage, a weaker prognostic indicator needed larger ''n'' to be able to show a statistical difference. In the whole population the relative risk of recurrence of between pathological stages pT2 and pT3 displayed only a 1.57-fold increase (22.8% vs. 35.9%) and required 500 cases to achieve the same accuracy of results. Stage pT2 prostate cancer is defined as organ confined disease while pT3 refers to extraprostatic extension, but still not invading bladder and rectum. Using 20 sets of 300 random cases gave a 25% chance (5/20) of an analysis showing false negative results. This requirement of relatively large sets is a result not only of the discriminating value of a prognostic marker (i.e., Gleason grade and pathological stage) but also of the low percentage of patients with prostatectomy that actually have biochemical recurrence. A low-recurrence rate requires a greater number of cases to prove statistical significance.

By comparison, when recurrence rates are higher, even 20 subjects followed for less than 3 years may be sufficient to test the prognostic value of p53 and Ki-67 staining in hepatocellular carcinoma in liver transplant patients (Guzman et al. 2005).

Selection of cases and TMA layout

The next step is collecting cases. This requires careful planning to maximize the number of projects that will use the TMA, appropriateness of controls, confirmation of diagnosis, grading, and staging of the cases, etc. If a project involves patients from a multi-year period, the researcher needs to ensure that patients accrued at the earlier phase of the study are comparable to those acquired near the end: for example, pre-PSA era prostate cancer patients versus PSA-detected era patients. The researcher must also keep in mind the full scope of the study; when it comes time to analyze the results, will a study regarding the patients' outcomes include only disease-specific death or any cause of death? Disease-specific cause of death as reported in death certificates is not reliable. Even prospective studies have difficulty with accuracy of death certificates. Only a small percentage of patients undergo autopsy in most countries. If a patient dies of pneumonia as a result of cancer-associated immunosuppression, many autopsy reports will attribute the cause of death to pneumonia, not to cancer. Countries where the autopsy rate is higher and where patient records are maintained by a central, socialized medicine environment usually have a higher rate of cases with known outcome than countries where the patients have more freedom to constantly change their medical care providers and are frequently lost to follow up. These considerations are obviously important when the goal of the TMA is to provide a tool for outcome studies that will use the Kaplan–Meier curve survival analysis because a high percentage of inaccurate endpoints will decrease the reliability of the results.

After the final number of cases available is known and all slides and paraffin blocks have been collected and reviewed by pathologists, the TMA layout can be created. Keeping track of randomly assigned hundreds of cores and replicates can be daunting and generate inaccurate records. The first consideration is how many cores to take from each case. Practically, for prostate cancer four 0.6 mm cores give greater chance of showing the same IHC result as a traditional ''large'' tissue section (Rubin et al. 2002). For diseases where large and relatively homogeneous tumors can be sampled, such as in cancers of the liver and ovary, a smaller number of replicates may be sufficient. The number of cores will also heavily depend on the percentage of cells that are positive for a specific marker but when building a TMA one has to plan for the markers that are not expressed in most cells, such as Ki-67 antigen. Surprisingly, TMA cores may show *better* correlation with outcome than ''large'', conventional sections. In a conventional tissue section a focal area of weak immunohistochemical staining may lead the investigator to classify the entire

case as positive. In TMAs, the sampling may be more representative by missing these areas.

Next, how large should the diameter of the cores be? As mentioned in Kononen et al. in this issue, 2.0 mm cores do not address concerns of tissue heterogeneity much better than 0.6 mm cores do. One exception would be tumors with abundant desmoplastic reaction of the stroma. These tumors may be better represented by 2.0 mm cores if the goal is to study epithelial cells and not stroma. There are projects where TMAs are inappropriate and conventional sections are recommended, irrespective of the size of the tissue cores used. An example would be a study of lymphoid and macrophage aggregates in carcinomas because the distribution of these cells is only focal in most cases, frequently forming aggregates.

Figure 1 is an example of such a layout. By writing a simple Excel program, one can randomly assign the study subjects to individual cells of the spreadsheet. Positions for control tissue cores should also be randomized and interspersed with the other cores. If all control tissues are in one area of the TMA, there will be inherent interpretation bias unless an instrument for image analysis is used. In addition, if all controls are in one area of the slide and there is unevenness of staining in the slide, results can be easily misleading. It is useful to arrange the cores in ‘‘city blocks’’ that reduce the possibility of error during construction and make visual core tracking during analysis easier. As seen in Fig. 1, at CPCTR we group cores in ‘‘city blocks’’ of 5×5 cores, but larger groups can be used, depending on how many cores one has to fit into one TMA paraffin block. For visual orientation purposes we place orientation tissue cores at the upper right corner of the slide, outside of the ‘‘city block’’ pattern. Sometimes a tissue core is not available to fill a position in the TMA distribution design, so it is acceptable to have blanks in the final recipient block.

Not all cores have to fit in one block. Even though 1,000 or more 0.6 mm cores can fit on a standard glass slide, this comes at a cost of crowding the cores and not being able to separate groups of cores into ‘‘city blocks’’. If a TMA is composed of more than one block, it is better to stain all the slides in one

6648288 903 Gleason= 9 4+5	1391891 298 HGPIN	1391891 298 Gleason= 6 3+3		9224759 400 Gleason= 9 4+5	8393468 414 Gleason= 8 4+4	46116710 06 Gleason= 7 3+4	9872541 567 Gleason= 9 4+5	3082106 906 Gleason= 5 2+3		9457124 113 Gleason =8 3+5	CELL LINE: LNCap
8928491 813 Gleason= 9 5+4	8733491 911 Gleason= 6 3+3	8733491 911 HGPIN		5350052 228 Gleason= 8 4+4	BPH - 5 2248738 214	31486113 76 Gleason= 7 3+4	2917944 176 Gleason= 7 3+4	6827904 003 Gleason= 7 4+3		BPH - 16 3066979 331	CELL LINE: LNCap
3216713 650 Gleason= 6 3+3	3216713 650 HGPIN	BPH - 18 3149117 675		8052174 271 Gleason= 8 4+4	8927508 071 Gleason= 8 3+5	67570891 91 Gleason= 6 3+3	6757089 191 HGPIN	3940932 352 Gleason= 9 4+5		7400441 834 HGPIN	CELL LINE: PC- 3
9520519 371 Gleason= 5 3+2	3415537 650 Gleason= 5 3+2	8782713 543 Gleason= 8 3+5		8912557 102 Gleason= 5 2+3	8912557 102 HGPIN	Normal from donor - 9 41547058 91	6544131 896 Gleason= 6 3+3	6544131 896 HGPIN		7400441 834 Gleason =9 4+5	CELL LINE: DU- 145
2994602 445 Gleason= 7 4+3	3689774 155 HGPIN	3689774 155 Gleason= 6 3+3		BPH - 1 2484517 713	BPH - 14 7649953 434	52601853 64 Gleason= 5 3+2	7315779 006 HGPIN	7315779 006 Gleason= 5 3+2		2865209 329 HGPIN	CELL LINE: PC- 3
8386907 442 Gleason= 9 4+5	6290660 541 HGPIN	6290660 541 Gleason= 6 3+3		5765253 778 Gleason= 7 3+4	5765253 778 HGPIN	17802248 95 Gleason= 5 2+3	Normal from donor - 13 2486430 743	9336666 967 Gleason= 5 3+2		2865209 329 Gleason =7 4+3	CELL LINE: DU- 145
7246497 924 Gleason= 9 4+5	8003118 596 Gleason= 6 3+3	8019623 662 Gleason= 7 3+4		3115988 785 Gleason= 9 4+5	3742013 631 Gleason= 7 3+4	16234289 86 Gleason= 9 5+4	4419720 538 HGPIN	4419720 538 Gleason= 6 3+3		8583079 036 Gleason =9 5+4	CELL LINE: DU- 145
1894589 598 HGPIN	2029934 536 Gleason= 8 4+4	7665347 121 Gleason= 5 2+3		5943185 192 Gleason= 9 5+4	5943185 192 HGPIN	47328186 43 Gleason= 7 3+4	4732818 643 HGPIN	9547110 791 Gleason= 8 4+4		6600059 907 Gleason =7 3+4	CELL LINE: LNCap

Fig. 1 Detailed map layout of a segment of TMA 1 available from the Cooperative Prostate Cancer Tissue Resource showing the 5×5 city block arrangement of tissue cores, the numeric code for each subject, Gleason score and grades for each case, interspersed cases of high-grade prostatic intraepithelial neoplasia (*HGPIN*) and normal tissues, benign prostatic hyperplasia (*BPH*) tissues and cell line controls

experiment in order to avoid between-run experimental variability.

Issues of human subjects protection in TMA design

In the example given by the layout in Fig. 1 the case numbers is not the patient's hospital identification numbers or surgical pathology accession numbers. These are randomly assigned code numbers that are only linked to the patient's hospital identification number through a third party that acts as the ''honest broker'' or trustee. This third party has access to the patient's history and follow-up but never has access to the patient's tissue sample or research results. This is in compliance with institutional review board (IRB) requirements and human subjects privacy rights legislation.

For projects performed in the USA, there is a very useful document issued on August 1, 2004 by the Office for Human Research Protections (OHRP) offering guidance on research involving coded private information or biological specimens (http://www.hhs.gov/ohrp/humansubjects/guidance/cdebiol.pdf). According to these guidelines, ''...under limited conditions, research involving *only* coded private information or specimens is not human subjects research...'' This means immense savings of time for researchers in terms of clearance with IRBs for use of TMAs. These official guidelines also bring a not well-publicized issue, the use of autopsy material or human research: ''...Human subject means a living individual...'' It should be noted however that ''...*obtaining* identifiable private information or identified specimens for research purposes constitutes human research...'' The responsibility of protecting identifiable private information lies with the investigators who build TMAs, especially before the samples are coded. At CPCTR, many of the hospitals supplying archived tissues enlist the help of their Tumor Registrars for the important position of honest broker. Tumor Registrars, by virtue of their training and function, are already very aware of confidentiality and human subjects' protection issues. They also are in position to obtain follow-up information on cancer patients as part of their daily hospital activities.

Additional TMA construction issues

Manufacturers will stress the advantages of using an automated microarrayer instrument as a time saver mechanism. Most of the time spent on constructing TMA is however on the preparative steps of the entire process, such as planning, obtaining human subjects institutional review approval, selecting cases for inclusion in the TMA, building the TMA layout spreadsheet, completing the patient outcomes database and, most of all, in reviewing each case for the areas to be included into the TMA. In order to locate the relevant tissue a pathologist must systematically examine the slides of each case under the microscope and circle in permanent marker the areas to be sampled. It is important to mark more than one representative area because some paraffin blocks may later be disqualified because their specimens are too thin. During the next step, the slides are overlaid on the paraffin-embedded donor tissue blocks of each subject's tumor and the markings are duplicated onto the paraffin. Finally, some of these markings must be disqualified by examining the three-dimensional geometry of the tumor. The tumor must not only be representative but also be deep enough into the donor paraffin block to appear in many cuts of the recipient TMA block. To investigate tissue depth in paraffin blocks, one of the CPCTR laboratories has used a Faxitron X-ray machine (Kong et al. 2006). Areas of necrosis should be avoided.

Once these preliminary steps have been completed, the next step is to construct the TMA. There are several types of tissue microarrayers available for constructing a TMA: manual, semi-automatic and automatic. Manual arrayers vary in complexity from simple skin biopsy punchers, to more sophisticated machines that have the advantage of perforating perfectly vertical punches on the recipient block. Commercial sources of inexpensive microarrayers include the TMA Builder (www.abcam.com, product number 1802) and Quick-Ray^{TM} (Woo-Ri Medic, Kent, WA, USA, www.woorimedic.com). More sophisticated instruments are available from Chemicon (Temecula, CA, USA) and Beecher Instruments (Silver Springs, MD, USA). At CPCTR we developed a semi-automatic stepper motor that adapts to the Beecher arrayer to punch wells in the recipient paraffin block, extract cylindrical tissue core sample from the paraffin-embedded donor block and finally release the tissue with a more precise equidistance of the cores (Matysiak et al. 2003).

Strategies for saving tissues

Additional problems can occur after the TMA block has been designed and built. Considering how precious each TMA cut is, every effort should be made to save

tissues. In order to get maximal number of unstained TMA slides from one TMA block it should be cut in one session, but this approach has problems of deterioration of the antigenicity of the specimen with time, as discussed below. The cutting should be extremely accurate to maximize the number of slides from one TMA. Try using a collimator to align block-face to the cutting plane of the microtome and if you decide to cut TMA slides in more than one session, always use the same microtome to cut a block.

Another strategy for saving tissues is to try using a tape-transfer system as recommended by the original papers on TMAs, but this method is still controversial because there is still a concern of whether the tape interferes with immunohistochemistry and in situ hybridization. More definitive research in this area is needed.

At CPCTR we save every cut of TMA blocks. If a section is technically unsatisfactory, either because it folds on itself or because a significant portion is lost, it is still saved for use as optimization slide for testing immunohistochemistry stains for example. We found that ~150–200 usable sections can be obtained from a single TMA block before 20% of the cores are lost in deeper section (Matysiak et al. 2003).

A common mistake in laboratories that have little experience with TMAs is to use actual TMA slides to find optimal conditions for staining/in situ hybridization. It is better to build a ''training miniarray'' with only 12–15 tissue cores for this purpose, using the same techniques that will be used in the actual TMA.

After the slides have been cut, it is desirable to stain those that you need for your study immediately as tissue deteriorates in even a few days due to aeration (Henson 1996; DiVito et al. 2004). The best way to protect slides against antigen deterioration is to combine paraffin coating with nitrogen storage (DiVito et al. 2004). However, if the number of cores is large enough the conclusions of the study may not be altered significantly even without preventive measures of antigen protection (Wild et al. 2005).

Choosing an appropriate antibody/probe

Choosing an appropriate antibody may be the most difficult and important part of TMA success. But with so many vendors, how does one choose? Read the literature carefully, searching for antibodies that have been successfully used in formalin-fixed, paraffin-embedded tissues before. Keep in mind that formalin fixation frequently masks the antigens and therefore antigen retrieval is necessary. Also look for antibodies that have been used in Western blots to rule out cross reactivity with other antigens.

An example of why it is so important to choose antibodies carefully was shown in the paper by Pallares et al. (2005) where four commercial antibodies, supposedly directed against PTEN markedly vary from each other. One of four antibodies showed nuclear positivity while the others showed cytoplasmic localization. Two of the antibodies reacted against PTEN transfected lines and one of the antibodies reacted against cells that were PTEN deficient. Similar problems have been reported for anti-COX-2 antibodies (Zha et al. 2001; Garewal et al. 2003). A thorough literature research is an important step before deciding which antibody to use.

TMA analysis

Of course, an image analysis system is desirable when analyzing the results of a study. However, if such an instrument is unavailable, scoring should be performed by several independent investigators in order to reduce bias. For simpler studies semi-quantitative evaluation can mean just giving each core a score from 0 to 3+. However, for more complicated studies, where there is considerable variability in cell staining within a single tissue core, an immunohistochemical staining index may be more appropriate in which each core is critically assessed for percentage of staining at each staining intensity score. For example, a core may be 60% ''score 4'' and 40% ''score 5'' on a scale where 5 is the maximal intensity. In this example, an index can be achieved in which $[(0.6 \times 4) + (0.4 \times 5)]$ gives a score of 4.4 in. scale that ranges between 0 and 20 (Davol et al. 2003). Other variations of staining indices should also be considered depending on the nature of the antigen expression (Umemura et al. 2004; Liu et al. 2005). Simple percentage of cells staining is the best approach for assessment of proliferation markers such as Ki-67. Certain clinically used markers in differential diagnosis such as keratins, HMB45 and CD20 do not even need a numerical score; their presence, even in a relatively small percentage of cells is sufficient for the core to be counted as positive.

An important but frequently ignored problem with quantitative and semi-quantitative analysis of immunohistochemical staining, whether in TMAs or in whole sections, is a long list of severe limitations of immunohistochemistry, most of them pre-analytical: Types of fixatives and fixation conditions such as pH, concentration, duration, and temperature; storage conditions; thickness of each section; staining methods

and reagents. Even under ideal conditions, there is over-fixation of the tissue surface and under-fixation of the central portion of the tissue specimen, which is eventually fixed by alcohol during tissue processing. During formalin fixation, methyl groups crosslinking may mask the desired epitope by unfolding the protein or by crosslinking with adjacent proteins. These problems are unlikely to be compensated by automated image analysis instrumentations. This inherent variability has to be taken into consideration when setting hopes on a truly quantitative analysis for the future.

When comparing results across different publications even more complex issues arise. For example, very sensitive antibody detection systems such as enzyme polymer enhanced methods may lead to shift in the distribution of cases with high and low expression of hormone receptors in breast cancer (Umemura et al. 2004).

Statistical analysis of the results

A biostatistician could suggest the most appropriate statistical test for data analysis. To visualize the survival distributions, most researchers use the Kaplan–Meier plot and then apply the Log-rank analysis to test for survival differences between groups. As mentioned above, this type of statistics requires a sufficient percentage of cases with adequate follow up, in order to avoid a large number of cases that have to be statistically censored in the analysis. For most other relationships, contingency tables and the chi-square test have been used to study immunohistochemical results, grade, stage, and nodal status.

TMA data management

Ideally, microphotographs of the stained cores together with the immunohistochemical score and statistical results should be compiled into one database for cross-checking the data as well as for future studies. Two data management tools are recommended. TMAJ is an open-source software that was developed at the Johns Hopkins University for managing TMA databases. The http://tmaj.pathology.jhmi.edu site includes a demonstration edition of this software. Alternatively, Stanford University offers a suite of downloadable software tools that can be accessed at http://microarray-pubs.stanford.edu/tma_portal/index.shtml and http://genome-www.stanford.edu/TMA/combiner/download.stml. More recently, the Stanford database management tools have been updated to facilitate data analysis for which tissues may have two or more scores per antibody and permits combination of data from multiple different TMAS (Liu et al. 2005).

Need for open access tissue microarray data exchange specification

Like gene expression studies, TMA experiments are data intensive, requiring substantial information to interpret, replicate or validate. To ensure inter-laboratory reproducibility, information describing the preparation of TMA blocks and slides need to be provided along with the TMA data records. CPCTR implemented an open access TMA Data Exchange Specification that allows TMA data to be organized in a self-describing XML document annotated with well-defined common data elements. The publication describing this specification provides sufficient information for the reproduction of the experiment by outside research groups, containing instruction and example of actual implementation (Berman et al. 2003, 2004).

Alternatives to building your own TMA

Notably, the time and attention necessary to design and construct a TMA may be excessive for a short-term or small-scale study. In that case, there are commercially available TMAs of varying tissue and types of cancer. These TMAs are mostly of the consecutive cases type, without knowledge of histological grade, stage, outcomes or demographic information such as age and ethnic group. Usually only the diagnosis is given. They are however useful for the purpose of testing tissue marker prevalence in specific types of cancer and control tissues. TMAs from academic institutions and from governmental agencies usually have more information on demographics, pathology review and outcomes. Because the latter are more difficult to produce, they are usually not available for collaborative studies or purchase unless the investigator has already demonstrated feasibility of the project, usually in the form of a publication with smaller number of cases using local resources.

Conclusion

Extensive planning and preparative steps are necessary before a TMA is built. Along the way several pitfalls and quality enhancement features can develop. Most of the issues involved in TMA construction and utilization are common to studies performed on standard

tissue sections, but there are issues that pertain to TMA alone.

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