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Tissue Microarrays in Clinical Oncology

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Abstract

The tissue microarray is a recently-implemented, high-throughput technology for the analysis of molecular markers in oncology. This research tool permits the rapid assessment of a biomarker in thousands of tumor samples, using commonly available laboratory assays such as immunohistochemistry and in-situ hybridization. Although introduced less than a decade ago, the TMA has proven to be invaluable in the study of tumor biology, the development of diagnostic tests, and the investigation of oncological biomarkers. This review describes the impact of TMA-based research in clinical oncology and its potential future applications. Technical aspects of TMA construction, and the advantages and disadvantages inherent to this technology are also discussed.

Introduction

The tissue microarray (TMA) was first described by Kononen in 1998 (1), and represents a high-throughput technology for the assessment of histology-based laboratory tests, including immunohistochemistry and fluorescent in-situ hybridization (FISH). Small cylindrical cores are extracted from standard formalin-fixed, paraffin-embedded tissue and arranged in a matrix configuration within a recipient paraffin block; thereby facilitating rapid analysis of hundreds of patient samples by a surgical pathologist. Since the introduction of tissue microarrays, this technology has been applied to the study of tumor biology, the assessment of novel molecular biomarkers and laboratory quality assurance. The TMA also serves as an excellent validation and translation platform for other types of high-throughput molecular research.

Tissue microarray construction and analysis

The initial identification and collection of tumor samples represents the greatest portion of the work associated with TMA construction. Samples need to be identified based on their availability in sufficient numbers to address the proposed scientific or clinical question. For example, prognostic studies will require a large number of cases with long-term outcome data to provide adequate statistical power. Similarly, a study investigating a novel diagnostic biomarker may require the identification of histologically-related entities to assess a biomarker's specificity. After archival tissue blocks are retrieved, a hematoxylin-and-eosin-

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stained slide needs to be reviewed by a pathologist to determine the best area of each tissue block from which to extract a core (Figure 1A).

Prior to TMA construction a sector map is designed (Figure 1B). The sector map specifies a location within the TMA for each core sample, and it is used to guide both assembly and subsequent scoring. For the physical construction of the TMA, a tissue microarrayer is required and available commercially (e.g. Beecher Instruments, Sun Prairie, WI, USA; Figure 2A). Basic models include two hollow needles and a block holder that operates on a manual basis. Based on the planned sector map, a core is removed from a blank paraffin “recipient” block. The second needle is then used to remove a core of representative tissue from the donor block (Figure 2B). The tissue core is then inserted into the previously created hole in the recipient block (Figure 2C). This process relies on the operator to properly calibrate and maneuver the needles and blocks; more automated systems use a computer to guide the operator and trace the coordinates of the recipient block. These steps are repeated for each donor block that is to be incorporated into the TMA. The construction phase is relatively fast, and a typical project involving 200 cases may take two months for case identification and collection, but only two days for array building.

Core sizes can range from 0.6 mm to 2.0 mm, with 0.6 mm most often used. The advantages of a smaller core include a lower incidence of lost cores during sectioning of the TMA, and a reduction in tissue material extracted from the donor block. With this size, at least 400 tissue cores can fit into a standard-sized recipient TMA block. For some heterogeneous lesions, such as Hodgkin lymphoma, a larger core may be preferable.

Sectioning of TMAs can be performed with a traditional microtome but this step requires an experienced histotechnologist. TMAs do not always section agreeably, particularly if multiple tissue types are included in the array. Each TMA section has significant value and commercial suppliers of TMAs charge well in excess of a hundred dollars per individual section (for arrays representing 50 – 100 cases, without clinical data). The number of sections that are available from one TMA block is dependent on the depth of the donor blocks, histotechnologist skill and the thickness of individual sections. The typical number of sections obtainable in practice ranges from 50 – 150. Immunohistochemistry or other applications can be performed on TMAs with minimal changes to standard protocols, although harsher antigen retrieval techniques or enzymatic digestions (e.g. deproteinization for FISH) may cause some tissue to detach from the TMA slide and be lost to analysis.

Scoring of the TMA can be performed under light microscopy, or the TMA can be digitally scanned and displayed on a high resolution monitor (Figure 3A). During scoring, each core in the TMA is assessed by a surgical pathologist, and the result is recorded on the sector map (Figure 3B and 3C). Scoring of the TMA is performed blinded to linked clinicopathological data, thereby reducing the potential for bias. As TMAs can include thousands of cases, scores from the sector map can then be merged with clinicopathological data with the aid of publicly available software, such as TMA-Deconvoluter(2).

Advantages of tissue microarrays

The staining of a few TMA sections in comparison to many more whole sections offers a clear benefit with respect to the use of laboratory reagents and technician time. As several research groups are now using TMAs representing in excess of a thousand tumors, this approach represents a major savings in scientific resources. In addition, there is also the benefit of decreased technical variability during the staining and interpretation process. The close proximity of cores also permits more rapid and consistent biomarker scoring by a surgical pathologist. In our experience, it requires approximately 15 working hours to score a single biomarker on a 4800 core tissue microarray series. Consequently, once construction of the

TMA is completed, an entire suite of biomarker studies can be accomplished in a few weeks, as opposed to several months with whole sections.

In the field of molecular epidemiology, TMAs offer some distinct advantages over other high through-put molecular techniques, including DNA microarrays and proteomics. The source material for TMAs is formalin fixed, paraffin-embedded tissues, and this is the most common method of preserving surgical specimens. Many hospitals are required to retain archival tissue blocks for at least 20 years, and thus source material for TMAs is readily available and is often linked to long-term outcome data. In contrast, DNA microarray and proteomics methods generally require fresh-frozen tissue. As a result, studies using these techniques must draw on much more limited supplies of existing frozen samples, or be conducted prospectively with special tissue handling protocols.

TMAs are used for in-situ molecular techniques, the most common of which is immunohistochemistry. Most other techniques, including DNA microarray, Western Blot, sequencing and PCR require processing and homogenization of tissue. Consequently, experimental data is detached from its morphologic context; and any biomarker measurement will include contributions from stroma, vessels, inflammatory cells, and normal surrounding tissue in addition to tumor cells. For TMAs, the core is selected from a microscope-guided specific area of the source block, ensuring that representative areas of tumor are sampled and areas of normal tissue or necrosis are avoided. Furthermore, TMA interpretation is performed with morphologic correlation – that is, in visualizing a TMA core, the histology is intact and the surgical pathologist is able to ignore non-cancerous elements and assign a biomarker score based specifically on the tumor cells present.

Among translational research approaches, TMA technology raises few ethical concerns. The methodology does not require any modified or additional procedures to be performed on patients, core extraction can wait until after a final pathology diagnosis is rendered, and the source blocks are minimally altered and remain useable for whole section analysis in rare cases where there is a clinical need to go back to the blocks at a later date. Following TMA construction, patient identifiers can be reversibly (where clinical outcome updates are anticipated) or irreversibly stripped from the sector map database, to anonymize the series.

Tissue microarrays in the study of tumor biology

TMAs permit the rapid assessment of individual molecular markers on large patient cohorts. This approach complements molecular screening and discovery studies by confirming results on large numbers of primary tumor cases. TMAs have been so employed, for example, in the initial papers characterizing new breast cancer oncogenes such as EMSY (3) and alpha-basic crystallin(4), and the recently-identified prostate cancer fusion oncogene TMPRSS2:ERG(5).

TMAs are similarly useful in characterizing the immunohistochemical profile of cancer subtypes. For example, two studies have applied a large panel of antibodies to determine differences in the molecular profile of BRCA1/2 breast cancer as compared to sporadic breast cancers. They found that BRCA1 tumors were hormone receptor and HER2 negative, p53 positive, and expressed a specific set of cell cycle antigens(6,7). There have also been studies that found new molecular markers in lower incidence cancers including nasopharyngeal cancer (8) and malignant melanoma(9). The discovery of new molecular markers provides some insight into the biochemical aberrations that lead to malignant progression, and in turn, this knowledge can contribute to the development of specific targeted therapies. In a prostate cancer study using TMAs, it was reported that there was co-expression of HIF, androgen receptor, and VEGF(10). Based on their results, the authors suggested that androgens may regulate VEGF levels (a key angiogenesis factor) through the activation of HIF, a transcription factor that regulates biological processes in response to hypoxia.

Progression TMAs are composed of cores demonstrating distinct stages of neoplasia (normal tissue, pre-invasive lesions, low and high grade invasive tumors, etc.) and they are also useful in the development of diagnostic assays. For some cancers, there is increasing evidence that screening can reduce cancer incidence and mortality (11,12), suggesting that early detection and treatment can improve patient outcomes. Screening procedures, including mammography and endoscopy, will often diagnose pre-malignant conditions. Progression TMAs are used to determine the expression of a biomarker in different stages of neoplasia, and can be used to identify markers of malignant transformation. Recent studies have reported on the expression of PCNA to distinguish esophageal adenocarcinoma from Barrett's esophagus(13), and have found that the loss of ANXII for prostatic epithelium is a marker for neoplasia(14).

Tissue microarrays for assessment of new diagnostic tools

In modern oncology, treatment decisions are critically dependent on accurate diagnostic pathology. Increasingly, molecular biomarkers are used in conjunction with conventional histology to improve diagnostic accuracy. Examples include the use of cytokeratin stain to localize micrometastases in sentinel lymph node biopsies for breast cancer, and the use of antibody panels to ascertain a tissue diagnosis for a metastasis of unknown primary origin. Primary unknown malignancies are particularly challenging as the biopsies obtained from metastatic sites are often poorly differentiated and difficult to diagnose based on histology alone, yet treatment options and prognosis could vary drastically depending on the exact diagnosis.

Large multi-tumor arrays can be used to estimate the diagnostic accuracy of a biomarker for a specific type of malignancy. One such study performed IHC analysis of FLI-1 on 4000+ tumors of various histologies. FLI-1 is a marker for Ewing family tumors but is also expressed in other small round cell tumors, and this study determined the sensitivity of FLI-1 to be 74% and the specificity 96%(15). Similarly, a large study of CD138 found that this glycoprotein, originally thought to be a specific marker of plasmacytic differentiation, was found to have widespread immunoreactivity in a 1700+ core multi-tumor array(16). Two recent TMA studies found p63, a marker of squamous differentiation, to be effective in distinguishing anal squamous cell carcinoma from low rectal adenocarcinoma(17), but in primary lung tumors it was found to stain both squamous cell and adenocarcinomas(18). Sarcomas and lymphomas are notoriously difficult to subtype by purely morphologic approaches, but new diagnostic biomarkers identified by gene expression profiling are being quickly tested, using TMA technology, against large numbers of histologically-similar neoplasms to determine their practical diagnostic value(19,20).

Tissue microarrays for assessment of prognostic and predictive value

In the clinical practice of oncology, therapeutic decisions regarding adjuvant treatment are often based on a clinician's estimate of recurrence risk, and the expected therapeutic gain from a specific treatment. More recently, clinicians have at their disposal prognostic models that are based on the retrospective analysis of large outcome databases. Examples include the Kattan nomograms for prostate cancer(21), and web-based prediction tool "Adjuvant! Online" for breast and colorectal carcinomas(22).

However, a significant drawback of these prognostic tools is that they rely upon the standard clinico-pathological features captured at the time of initial data collection. Thus, patient cohorts that have sufficiently mature outcome data for model-building often lack important biomarker information that would now be routinely collected. One example is the absence of HER2 status from the SEER data used in "Adjuvant! Online." Of course, it is now known that HER2 status is an important prognostic and predictive biomarker in breast cancer. The ability to perform additional laboratory investigations on these large cohorts would be of great clinical value.

Because TMA construction uses standard archival tissues, this technology is ideally suited for study or re-analysis of such valuable patient cohorts.

With DNA microarray technology, the expression of thousands of genes can be analyzed efficiently, and the pool of potentially useful prognostic biomarkers is rapidly increasing. However, high cost and limited tissue availability prevent the application of DNA microarray technologies to archival tissue libraries. The construction of a TMA requires only a small core from an archival block, and each core can then be used to assess multiple biomarkers – thus, this technology is appropriate for use on tissue banks with limited sample availability. The biomarker data acquired from TMA analysis can then be interpreted in the context of clinicopathological and outcome data for large patient cohorts. Furthermore, because TMA technology is based on widely available IHC and in situ hybridization techniques, results can be readily validated in other laboratories. However, one disadvantage of TMA studies is the need to assess each biomarker separately – thus, TMAs are not ideal for use as a discovery tool.

Many research groups are employing a two-step, discovery and validation approach to finding clinically useful biomarkers(23). The discovery phase involves the screening of a large number of molecular markers and finding those associated with clinically relevant endpoints – DNA microarrays are particularly useful for identifying potentially useful biomarkers. However, these studies are prone to false discovery as thousands of genes are assayed on relatively small patient cohorts (24). In the validation phase, where appropriate antibodies are available, the protein products of top candidate genes can be assayed in large, independent patient cohorts using TMAs. A study demonstrating the prognostic value of FoxP1 in diffuse large B-cell lymphoma exemplifies this approach(25).

DNA microarrays can also identify tumor subgroups based on gene expression; one example is the discovery of an intrinsic classification of breast cancer(26,27). Gene expression profiling of a limited number of breast cancer tumors revealed 5 subtypes with prognostic relevance; these results have been reproduced in subsequent studies on independent patient cohorts. A panel of four immunostains derived from this profile was validated on TMAs, and was found to identify IHC-defined subgroups that were similar to the intrinsic classification, and had prognostic relevance(28).

In recent years, there have been a large number of studies that report on the prognostic value of individual or combinations of biomarkers, for particular cancer types. Studies include tumor sites for which there already exist strong prognostic models, such as breast and colorectal cancer, but also for malignancies in which prognosis is still based on TNM staging, including carcinoma of the head and neck(29,30) and lung cancer (31,32). For less common malignancies and for those where we lack good prognostic models, TMA technology has great potential for benefit. Examples that have particular relevance to the radiation oncologist are studies demonstrating that VEGF and Cox-2 are prognostic biomarkers in early stage laryngeal cancer treated with primary radiotherapy(33,34).

In addition to the development of prognostic models, biomarker research is also being used to develop predictive tests. While there are tests for specific targeted therapies such as trastuzumab (for HER2 positive breast cancer) and imatinib (for c-kit positive gastrointestinal stromal tumors), there is also some evidence that molecular markers can predict for response to conventional adjuvant treatments(35,36). Recently, a research group from the Royal Marsden assembled a large TMA comprised of patients enrolled in the ATAC study (Arimidex, Tamoxifen Alone, or in Combination), a randomized clinical trial that compared adjuvant tamoxifen to anastrozole for ER positive breast cancer. In the most recent published report of this study, anastrozole provided a statistically significant improvement in disease free survival

(37). The purpose of the correlative TMA study is to discover molecular markers that predict for tamoxifen resistance or response to anastrozole(38); initial results suggest that the benefit of anastrozole over tamoxifen is independent of quantitative ER and PR expression, and Her2 status (39).

Tissue microarrays in quality control and clinical practice

While TMA studies are an important tool for the validation of novel biomarkers, most molecular epidemiology studies are derived from a single institution; and even if the tumor samples are collected from multiple sites, sample processing and data collection and analysis are performed centrally. There are multiple reports in the medical literature of conflicting results on the prognostic or predictive value of a novel biomarker. One example is Cyclin E in breast cancer, for which one research group reported strong prognostic value on multivariate analysis(40). Some studies confirmed these findings, but others failed to reproduce these results (41,42). Inter-institution variations in laboratory technique, specimen handling, patient populations, and criteria used for immunostaining interpretation will certainly contribute to differences in results(43). While this presents some difficulties in biomarker research, inter-laboratory variations in a clinical test can profoundly affect patient care.

In order to effectively use a biomarker assay in the clinical setting, the test must be shown to be consistent and reproducible. TMAs can be applied in quality assurance studies to assess inter-laboratory variations. Unstained sections from a TMA can be sent to multiple laboratories; ideally, this TMA would consist of samples collected from a number of different institutions. Each laboratory then processes and scores the TMA section independently. The results from each laboratory can be assessed for concordance, and compared to whole section results. This process was recently performed for two important predictive biomarkers, HER2 in breast cancer (44) and CD117 (c-kit) in gastrointestinal stromal tumors(45) (Figure 4), and for a number of diagnostic biomarkers in diffuse large B-cell lymphoma(46).

TMAs can also be used to improve existing biomarker assays. For example, a recent study compared the diagnostic effectiveness of two antibodies in determining estrogen receptor status in breast cancer (47). Both antibodies were applied to a large 4000+ case TMA, and the results of staining were compared to the original biochemical test for ER that was performed at the time of diagnosis. While maintaining specificity and equal positive predictive value, the more recently developed rabbit monoclonal antibody (SP1) was determined to be 8% more sensitive than the common clinically-used mouse monoclonal antibody (1D5) (Figure 5).

While TMAs have an important role in research and quality assurance, they are not generally used in clinical laboratory testing. However, for certain common biomarker assays, such as ER and HER2 for breast cancer, TMAs present a more cost-efficient approach to routine testing. Two studies have reported high concordance between TMA scores and whole section results for HER2 testing (48,49). Furthermore, a centre in Italy reported on a study using TMAs in ER, PR and HER2 testing on breast cancer, they found that TMA results were concordant with whole section results, and were effective in reducing reagent use, and technician and pathologists' time(50).

Validation of tissue microarray analysis

The most frequent criticism of TMA technology relates to the small size of each tissue core – there is concern that due to tumor heterogeneity, biomarker scores obtained from small TMA cores will not accurately reflect scores obtained from whole sections(43,51,52). It should be noted, however, that although whole section analysis is a “gold standard” for in-situ tests, whole sections themselves represent just a small portion of a tumor; and indeed core needle, punch and bite biopsies used for primary diagnosis often do not contain much more diagnostic tissue

than a TMA core. The concern of tumor inhomogeneity is partially addressed by visualizing the tumor region from which a TMA core is extracted; in addition, numerous studies have now been published validating the use of TMAs in various tumor sites, including breast(53), lung (54), and colorectal carcinomas(55). Using one to four cores per source block most TMA validation studies demonstrate good concordance between biomarker scores obtained from TMA and those obtained from whole section. Indeed, whole sections can be overinterpreted, especially for biomarkers with focal staining (53). In general, the biomarkers of true clinical value will display relatively uniform staining in tumors, and TMAs are certainly adequate in such cases.

A significant technical problem associated with TMA studies is tissue loss during sectioning, transfer, and staining (56). Some reports estimate that the rate of core loss is 10–30% due to technical causes alone. Furthermore, due to variations in preparation and storage of source blocks, tissue quality can also affect biomarker results. One study demonstrated loss in antigen immunoreactivity with increasing time between sample preparation and staining (56). It is reasonable to assume that all TMAs will contain a fraction of non-reactive cores that will be uninterpretable or false negatives during analysis.

The combination of sampling error during core extraction, core loss during slide preparation, and non-reactive cores leads to missing biomarker data, and under-estimation of the true incidence of a molecular marker. Missing biomarker data may necessitate the exclusion of cases and a consequent loss of statistical power during analysis. This is especially a problem for studies involving multiple biomarkers, where most statistical analyses require complete biomarker data for each case. Both the issues of missing data and under-estimation of biomarker incidence can be partly addressed by extracting multiple cores from each source block.

During TMA construction, the number of cores extracted per source block must be decided. One study addressed this issue by constructing four replicate tissue microarrays from 553 breast cancer specimens(53). For ER, the estimated incidence of positive cases from using a single core was almost identical to the incidence obtained from whole section analysis. In contrast, for PR, analysis of any one of the replicate arrays underestimated the incidence. However, if data from additional replicate arrays were combined, the TMA incidence for PR approached that obtained from whole section staining. With respect to missing data points, analysis of a single TMA was associated with 25–30% uninterpretable cores, combined analysis of three TMAs reduced data loss to 5–7%, and there was a marginal additional benefit from using all four replicate TMAs.

Of course, the use of multiple cores can rapidly increase the workload associated with TMA preparation and biomarker scoring, especially in large series. Despite improved data retention with the use of multiple cores per case, the authors of the previous study found that analysis of a single TMA (one core per case) produced the same prognostic effect for ER and PR as analysis of whole section staining. They conclude that in sufficiently large patient cohorts, use of single core TMAs can reproduce important clinical associations. This is a particularly important conclusion as it underscores the intent of most TMA studies: to discover or confirm important clinicopathological associations. As these studies are surveying entire patient cohorts, loss of some individual data points is acceptable. In most cases, false negatives and lost cores will occur randomly, and statistical analyses can easily be performed on cases with missing biomarker data to exclude systematic bias.

Conclusions and future directions

The most important step in initiating a TMA study is the identification of appropriate patient cohorts with accessible archival tissue materials. Prognostic studies require patient data that is

thorough, complete, and includes long term follow-up. Diagnostic studies may require locating source blocks from multiple tumor types and tissues in various stages of malignant progression.

In laboratory medicine, TMAs already play an important role in quality assurance and the characterization of new antibodies for IHC. There have now been numerous large molecular epidemiology studies using TMAs, but to this point, the impact to clinical oncology has been relatively limited. Prior to clinical application, biomarkers still need to be validated in multiple studies by independent groups. Although this process could be lengthy, it has potential to significantly affect prognostic estimates and clinical practice.

The next, critical development in cancer therapeutics will be predictive assays and targeted therapies. The potential of TMA technology is now more widely recognized and source blocks are being prospectively collected during clinical studies, eliminating the need to conduct tedious searches for archival tissue blocks. Indeed, planned construction of TMAs from surgical materials involved in cancer trials can greatly facilitate subsequent correlative science projects. The tissue microarray is a powerful tool in translational research, and its potential extends beyond oncology – it is an accessible, cost-efficient, and reliable means for the assessment of molecular markers in a clinically practical format.

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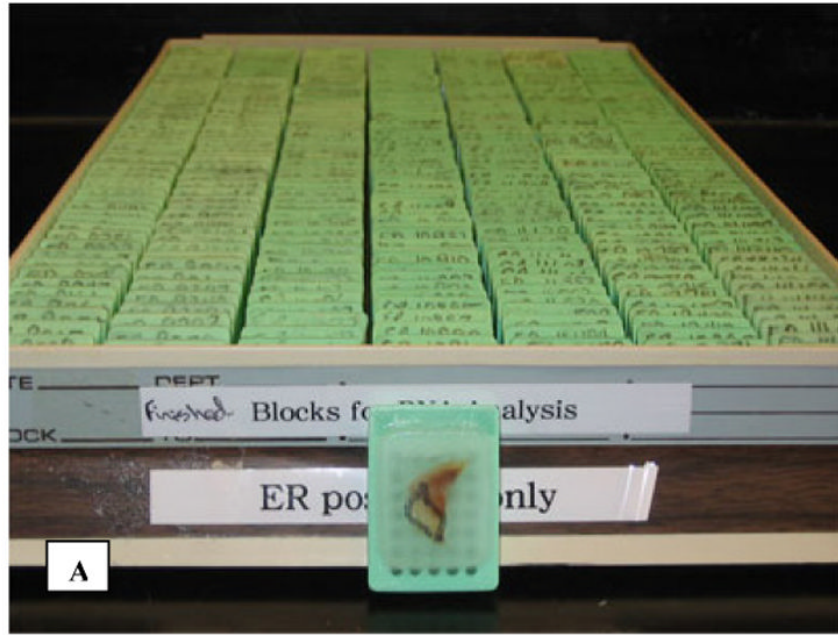
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sector	1	2	3	4	5	6	7	8	9	10	11	12	sector	1	2	3	4	5
1													1	169	170	172	173	174
2	24	23	22	21	5	19	18	17	16	15		13	2	192	191	190	189	188
3	25	26	27	28	29	30	31	32	33	34	35	36	3	193	194	195	196	197
4	48	47	46	45	44	43	42	41	40		38	37	4	216	215	214	213	212
5	49	50	51		53	54	55	56	57	58	59	60	5	217	218	219	220	
6	72	71	70	69	68	67	66	65	64	63	62	61						
7	73	74	75	76	77				81	82	83	84						

sector	1	2	3	4	5	6	7	8	9	10	11	12
1	96	95		93	92	91	90	89	88	87	86	
2	97	98	99	100	101	102			105	106	107	108
3	120	119	118	117	116	115	114			111	110	109
4	121	122	123	124	125	126	127	128	129	130	131	132
5	144	143	142	141	140	139	138	137	136	135	134	133
6	145	146	147	148			151	152	153	154	155	156
7	168		166	165		163	162	161	160	159	158	157

Figure 1. Tissue microarray planning. (A) Archival blocks are assembled and a surgical pathologist reviews the H&E slide for each case. The pathologist then circles the area of the block, localizing a representative tumor region from which a core will be extracted. (B) A sector map is designed; this is a grid that specifies a location within the TMA for each core sample. The sector map is then used to guide TMA construction and subsequent scoring, and it links biomarker scores to clinicopathological data on each case.

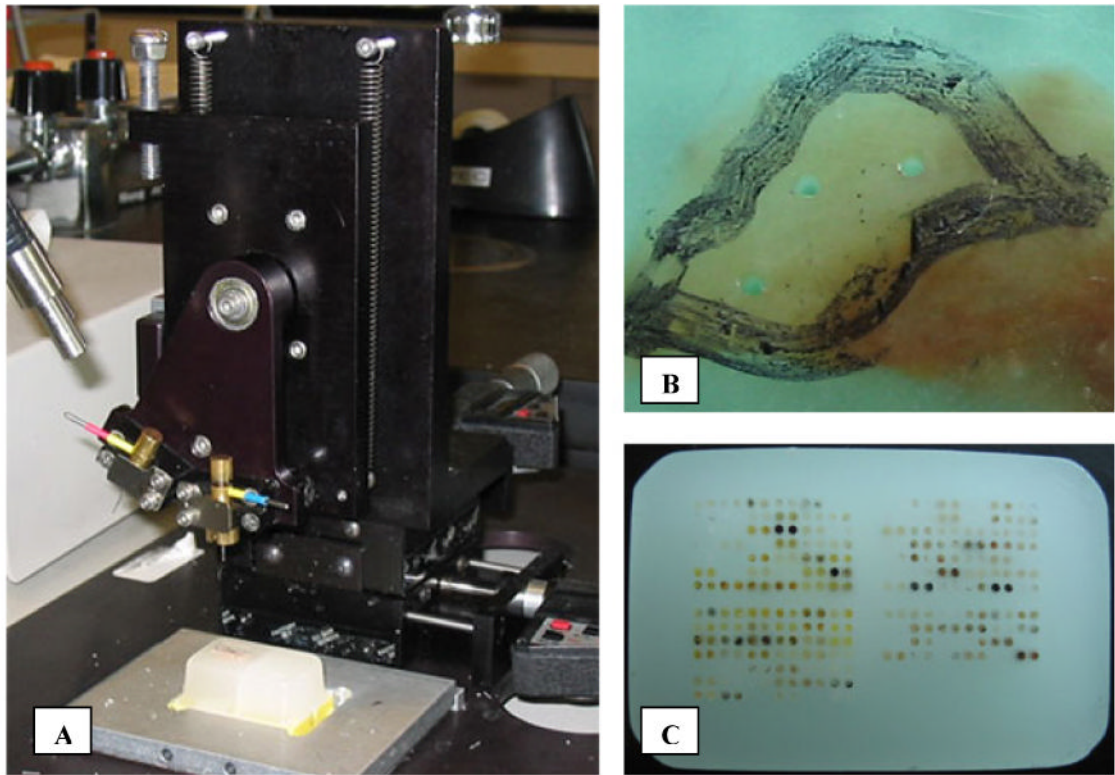


Figure 2. Tissue microarray construction and staining. (A) Beecher Instruments Microarrayer. The main components of the microarrayer are 2 hollow needles with stylets, a magnetic paraffin block holder, and positioning micrometers. (B) “Donor” block following extraction of triplicate 0.6 mm cores. The representative tumor region has been circled with a marker, and the cores were extracted from this area. (C) Completed TMA “recipient” block comprised of 300 cores.

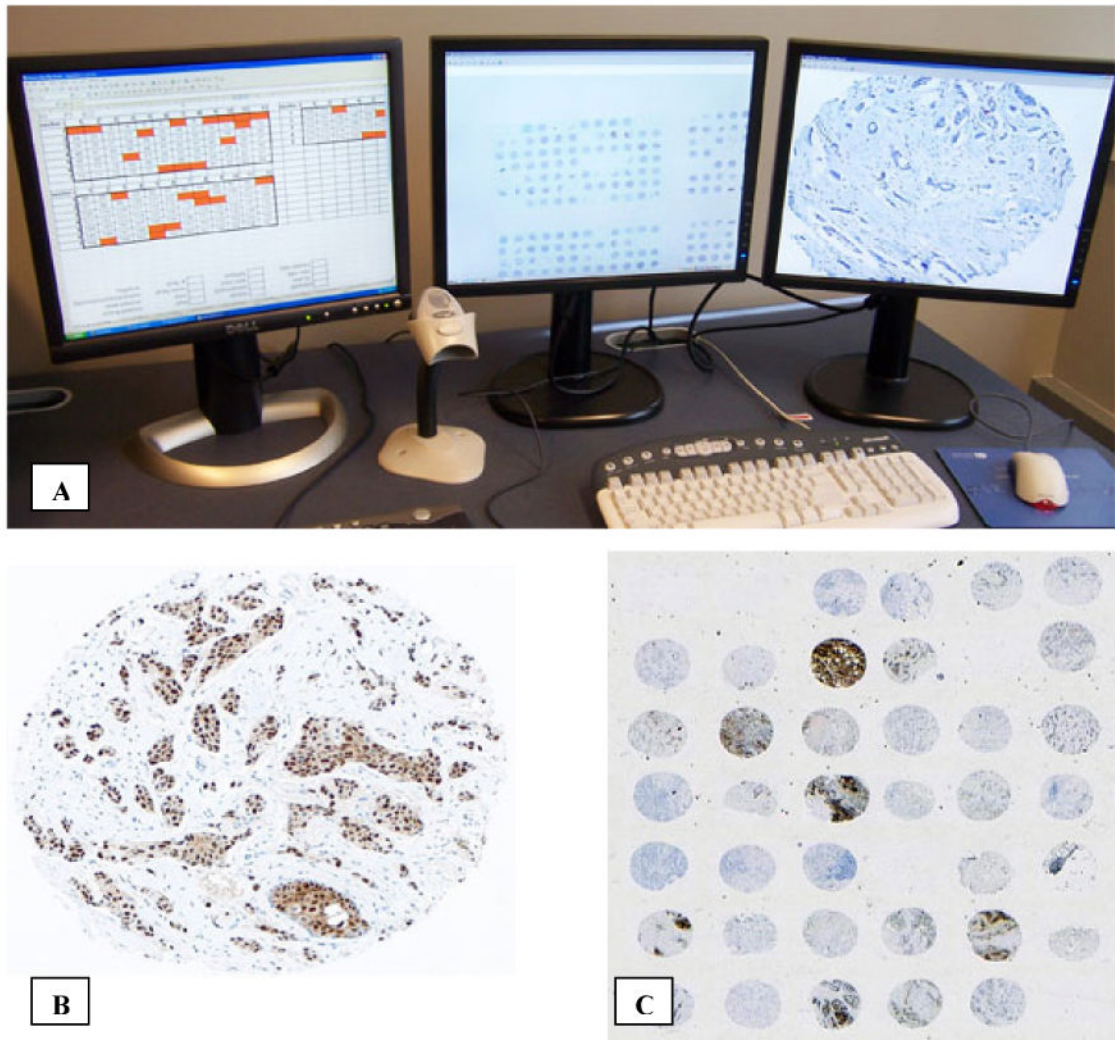


Figure 3. Scoring the TMA. (A) Example of a TMA scoring workstation with multiple displays. The TMA is best displayed concurrently at low magnification for orientation and unambiguous core assignment, and high magnification for scoring. (B) Sample of a TMA core under high magnification. This is a core from a breast adenocarcinoma that has been stained for estrogen receptors. (C) The TMA under low magnification.

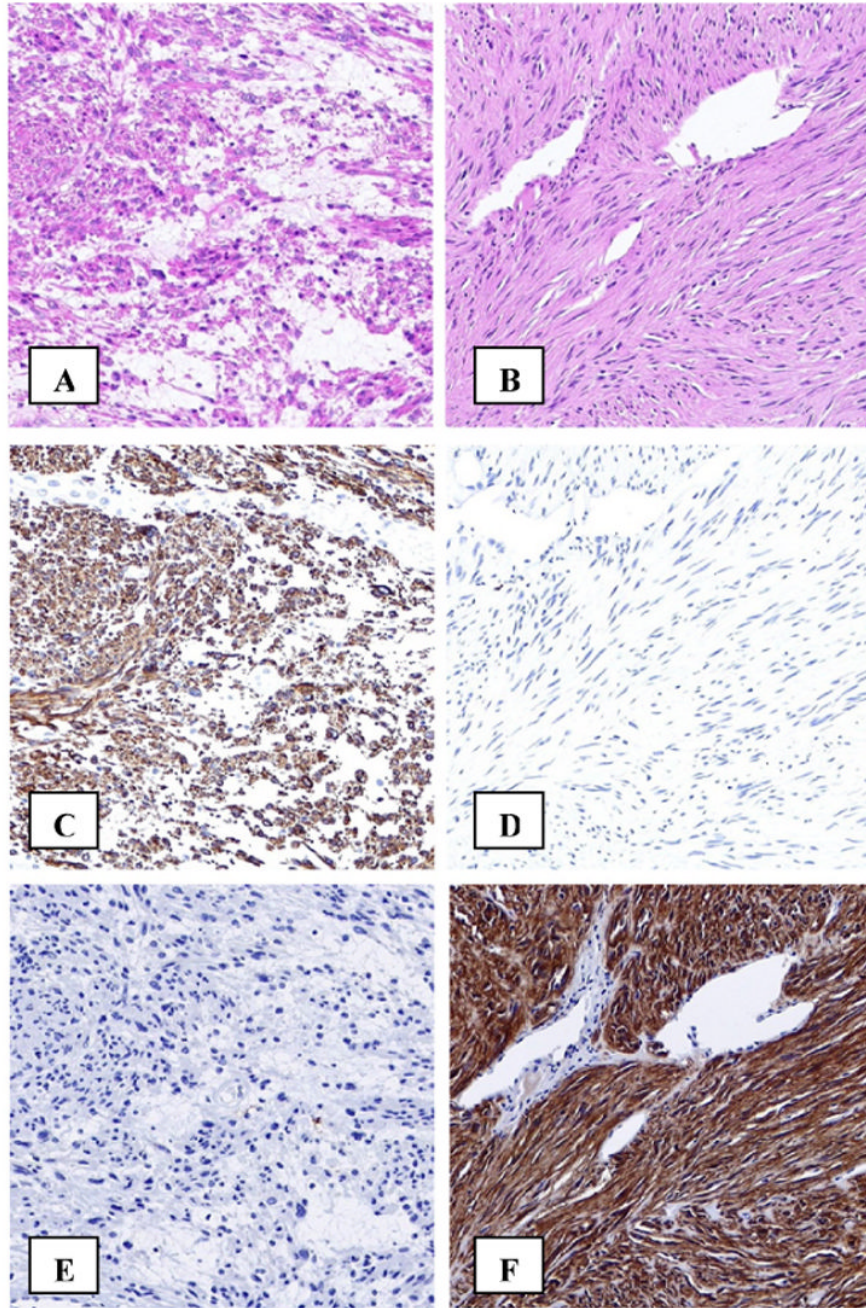


Figure 4. Immunohistochemistry can improve pathological diagnosis. Immunohistochemistry plays an important role in differentiating between gastrointestinal leiomyosarcoma (A) and gastrointestinal stromal tumor (GIST) (B). Desmin immunostaining is positive in leiomyosarcoma (C) but negative in GIST (D). Conversely C-Kit immunostaining is negative in leiomyosarcoma (E) and positive in GIST (F).

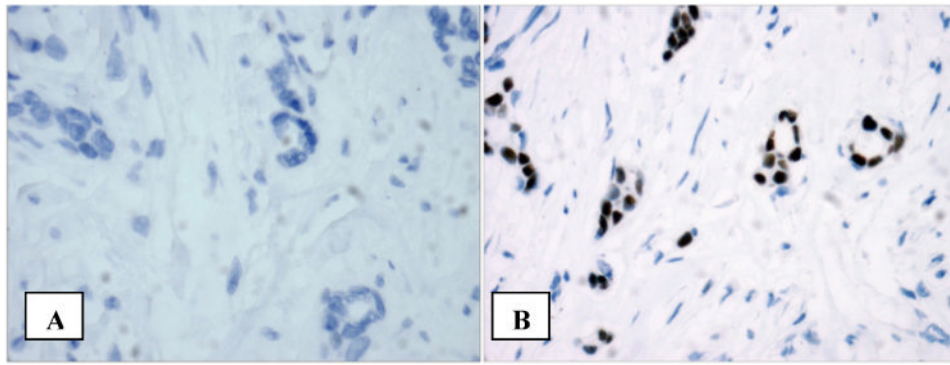


Figure 5. TMAs in laboratory medicine. Using a large tissue microarray, 2 estrogen receptor (ER) antibodies were assessed on more than 4000 invasive breast cancer tumors. It was found that the novel SP1 antibody was 8% more sensitive and equally specific as compared to the more commonly used 1D5 antibody. This breast cancer tumor would be classified ER negative with 1D5 (A) but is strongly positive with SP1 (B).