Int. J. Cancer: **94,** 1–5 (2001)

Publication of the International Union Against Cancer

TISSUE MICROARRAYS (TMAS) FOR HIGH-THROUGHPUT MOLECULAR PATHOLOGY RESEARCH

Antonio NOCITO[1], Juha KONONEN[2], Olli-P. KALLIONIEMI[3] and Guido SAUTER[1]*

[1]*Institute of Pathology, University of Basel, Basel, Switzerland*
[2]*Laboratory of Cancer Genetics, University of Tampere, Tampere University Hospital, Tampere, Finland*
[3]*Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA*

A rapidly increasing number of genes are being suspected to play a role in cancer biology. To evaluate the clinical significance of newly detected potential cancer genes, it is usually required to examine a high number of well-characterized primary tumors. Using traditional methods of molecular pathology, this is a time consuming endeavor rapidly exhausting precious tissue resources. To allow for a high throughput tissue analysis we have developed a "tissue chip" approach (Kononen et al., Nat. Med. 1998;4:844–7). Using this tissue microarray (TMA) technology, samples from up to 1,000 different tumors are arrayed in one recipient paraffin block, sections of which can be used for all kind of *in situ* analyses. Sections from TMA blocks can then be utilized for the simultaneous analysis of up to 1,000 different tumors on the DNA, RNA or protein level. TMAs allow a high throughput molecular analysis of thousands of tumors within a few hours. All currently available data have suggested that minute arrayed tissue specimens are highly representative of their donor tissues. There are multiple different types of TMAs that can be utilized in cancer research including multi tumor arrays (containing different tumor types), tumor progression arrays (tumors of different stages) and prognostic arrays (tumors with clinical endpoints). The combination of multiple different TMAs allows a very quick but comprehensive characterization of biomarkers of interest. We anticipate that the use of TMAs will greatly accelerate the transition of basic research findings to clinical applications.

Key words: *tissue microarrays; high throughput; pathology; immunohistochemistry; prognostic markers*

Development and progression of cancer is caused by a malfunction of genes. Biological and clinical properties of tumors, such as invasion, growth, metastasis and response to therapy, are all influenced by an altered expression of dozens if not thousands of genes. Most of the existing literature on the involvement of genes in cancer is based on the 6,000 known or "named" genes. However, these constitute just a small fraction of the estimated 30,000–50,000 genes in the human genome. The progress in human genome sequencing as well as the development of powerful functional genomic techniques, such as cDNA microarrays[1,2] and proteomics has dramatically changed our ability to identify genes and proteins whose differential expression is associated with cancer development. These techniques may in the future have a significant role in supplementing routine diagnostic assessment and classification of cancer. They may also facilitate definition of diagnostically or therapeutically significant subgroups of cancers. Individual genes and proteins that are identified may yield specific molecular targets for development of novel diagnostic or therapeutic strategies. In the future, development of new diagnostics and therapeutics will be concurrent. Therapeutic agents are increasingly developed against specific target genes and proteins, and a diagnostic method is needed to define those patients whose tumors express the therapy targets and are therefore most likely to benefit from the treatment.

Before any novel gene or protein targets are selected for diagnostic and therapeutic development, they need to be validated in large-scale tumor materials. Target validation can be accomplished using mRNA *in situ* hybridization or protein immunostaining on clinical tissue samples. These *in situ* techniques help to define the role of candidate molecular targets in the tissue context, such as determining the specific cell types that express the targets (tumor, stromal, adjacent normal tissue, infiltrating leukocytes, *etc*.), the subcellular distribution of the targets and the clinico-pathologic characteristics of the patients whose tumors express a candidate marker of interest. However, compared with the high-throughput genomics technologies used in gene identification, traditional microscope-based tissue analysis is labor-intensive, expensive, require extensive user interaction and has a low throughput.

There is now substantial interest in developing high-throughput molecular pathology techniques. Molecular pathology will be an essential early step in the conversion of novel genomic discoveries to future diagnostic and therapeutic tools. However, using conventional techniques, the evaluation of dozens or hundreds of newly detected candidate markers in tissues can take months or years and would be expensive. Moreover, the analysis of multiple markers from consecutive sections of tumors can rapidly lead to the loss of precious tissue materials. The number of sections that can be cut from a tumor block for *in situ* analyses limits the number of evaluable biomarkers to ≈200–300. Therefore, there is an urgent need to improve the speed and cost-effectiveness of molecular pathology research as well as to ensure that tissues are preserved for future uses.

TISSUE MICROARRAYS (TMAS)

Our recently developed TMA technology has the potential to significantly accelerate *in situ* studies of tissue specimens, to explore associations between molecular changes and clinicopathologic information and to ensure preservation of unique and precious research materials.[3] In this technology, tiny tissue cylinders are acquired from hundreds of different primary tumor blocks and arrayed into a single "recipient" paraffin block at high density (Fig. 1). Sections from such tissue microarray blocks can then be used for simultaneous *in situ* analyses of up to 1,000 tissue specimens either at the DNA, RNA or protein level. The concept of bringing multiple tissue specimens into a single slide was introduced in the form of a multi-tissue or "sausage" block technique more than 10 years ago.[4] The TMA technology uses a different sampling strategy and provides a higher level of sophistication by using small, regularly sized and shaped specimen cores that are arrayed with a precision instrument at a high density. Precise *x-y* positioning of the specimens provides a basis for automation of array construction, as well as automated image acquisition and storage.

The sampling for TMAs maximizes the number of parameters that can be analyzed from each patient specimen, and minimizes the damage to the donor blocks. Figure 2 illustrates how dozens of

*Correspondence to: Institute of Pathology, University Hospital, Schönbeinstrasse 40, 4003 Basel, Switzerland. Fax: +41-61-265-3194.
E-mail: Guido.Sauter@unibas.ch

Received 5 January 2001; Revised 28 March 2001; Accepted 4 April 2001

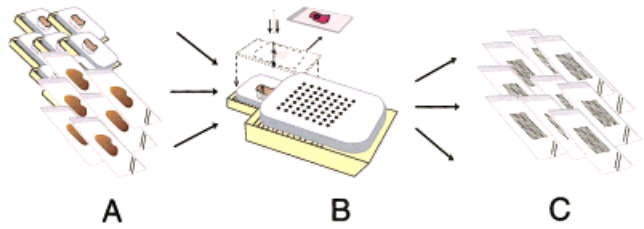

FIGURE 1 – Tissue-microarray construction: (*a*) Useful slides and paraffin blocks of possible donor tissue are collected from the archive. (*b*) Tissue core biopsy of 0.6 mm in diameter is punched from a pre-selected region of a donor block using a thin-wall stainless steel tube. A hematoxylin&eosin-stained section overlaid on the surface of the donor block is used to guide sampling from representative sites in the tissue. The tissue core is transferred into a pre-made hole at defined array coordinates in the recipient block. (*c*) Sections from a tissue microarray block are ready to be used for simultaneous *in situ* analyses.

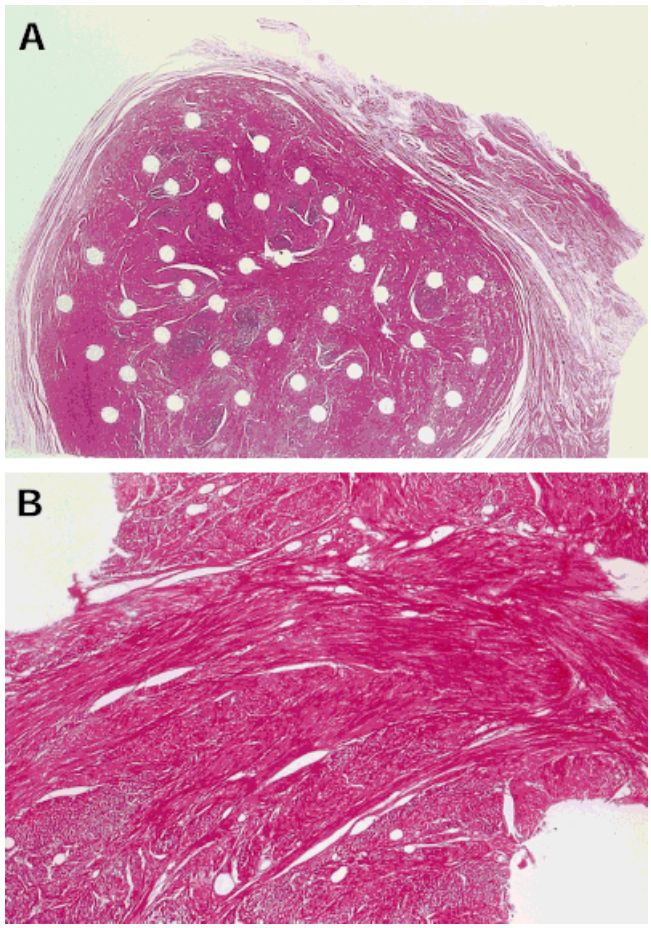

FIGURE 2 – (*a*) Overview of a tissue block after multiple sampling-procedures. (*b*) Interpretability of sections is not compromised after dozens of punches have been taken from 1 tissue block. Original magnification for (*a*) ca. 5×; ca. 50× for (*b*).

punches can be taken from a tumor without compromising interpretability. This is critical in the case of tissue blocks from diagnostic tissue specimens that need to be retained for potential further diagnostic evaluation (such as immunohistochemistry) later on. With the TMA technology, pathologists can more readily sample tissues for researchers without compromising future diagnostic needs and without removing the original tissue block from the diagnostic laboratory.

Sampling once from a single site in each tumor generates 1 TMA block, but in most cases uses only a minor fraction of all available tissue. Therefore, each tissue can be readily sampled dozens of times, generating dozens of replicate TMA blocks, each of which can be sectioned up to 200–300 times. Therefore thousands or tens of thousands of replica TMA slides can be obtained from a set of clinical tissue specimens.

APPLICATIONS OF TMAS

TMAs can be used for various molecular analyses that can also be performed on regular tissue sections, including immunohistochemistry, fluorescence *in situ* hybridization (FISH) and mRNA *in situ* hybridization. In general, the same hybridization and staining protocols can be used as for large sections. Furthermore, the TMA section thickness can be varied according to the question that is being asked in a particular study. For example, in FISH analysis, some investigators may prefer slightly thicker TMA sections in case of deletion analysis than in studies in which gene amplification is being evaluated. Examples of the application of TMA sections in various molecular detection schemes are shown in Figure 3.

Virtually all kinds of tissues or cells can be converted to a microarray format. Therefore, the range of potential TMA applications covers all fields of microscopic analyses of tissues and cells. In the case of cancer research, TMAs significantly facilitate the ability of basic scientists to extend *in vitro* studies of genes, proteins and signaling pathways to the *in vivo* situation. TMAs provide basic cancer research laboratories access to highly characterized tissues, reviewed by an expert pathologist, often containing associated clinico-pathologic, demographic or even survival and treatment outcome information. This access will facilitate attempts to link novel genes with clinical end points. Although most of the applications of the TMA technique described in this review come from cancer research, it is likely that the technology will be equally powerful in other fields of research, for example in inflammatory, cardiovascular or neurological diseases.[5]

In the past 2 years, the Institute of Pathology at the Basel University Hospital has constructed TMA blocks covering a large number of tissue types. These TMAs belong in 3 different categories that we have termed multi-tumor, tumor progression and patient outcome arrays.

Multi-tumor TMAs

Multi-tumor TMAs are composed of samples from multiple histological tumor types or organ sites. These arrays can be used to screen different tumor types for the expression of a molecular target of interest. Our first multi-tumor TMA contained 397 samples from 17 different tumor types, including specimens from the most common cancer types. This TMA was used to screen for amplifications of the oncogenes *MYC*, *HER2* and *CCND1* using FISH.[6] The comparison of the results of this TMA analysis with the previous literature suggested a high concordance, supporting representativity of the TMA results. A recent multi-tumor TMA contained 4,788 samples (distributed among 10 TMA blocks) of 130 tumor types including 354 normal tissues and 753 metastatic cancers. Sections from these arrays provide a template for a comprehensive analysis of the expression of molecular markers in various normal tissues as well as across the spectrum of neoplastic disease in man. Table I provides an example of p53 IHC data obtained for different gastrointestinal tumors in a multi-tumor TMA.

Tumor progression

TMAs can be used to study molecular alterations in different stages of tumor progression within a given organ, such as brain,[7] breast,[3] urinary bladder,[8,9] kidney[10] or prostate.[11,12] For example, a prostate cancer progression TMA was constructed containing

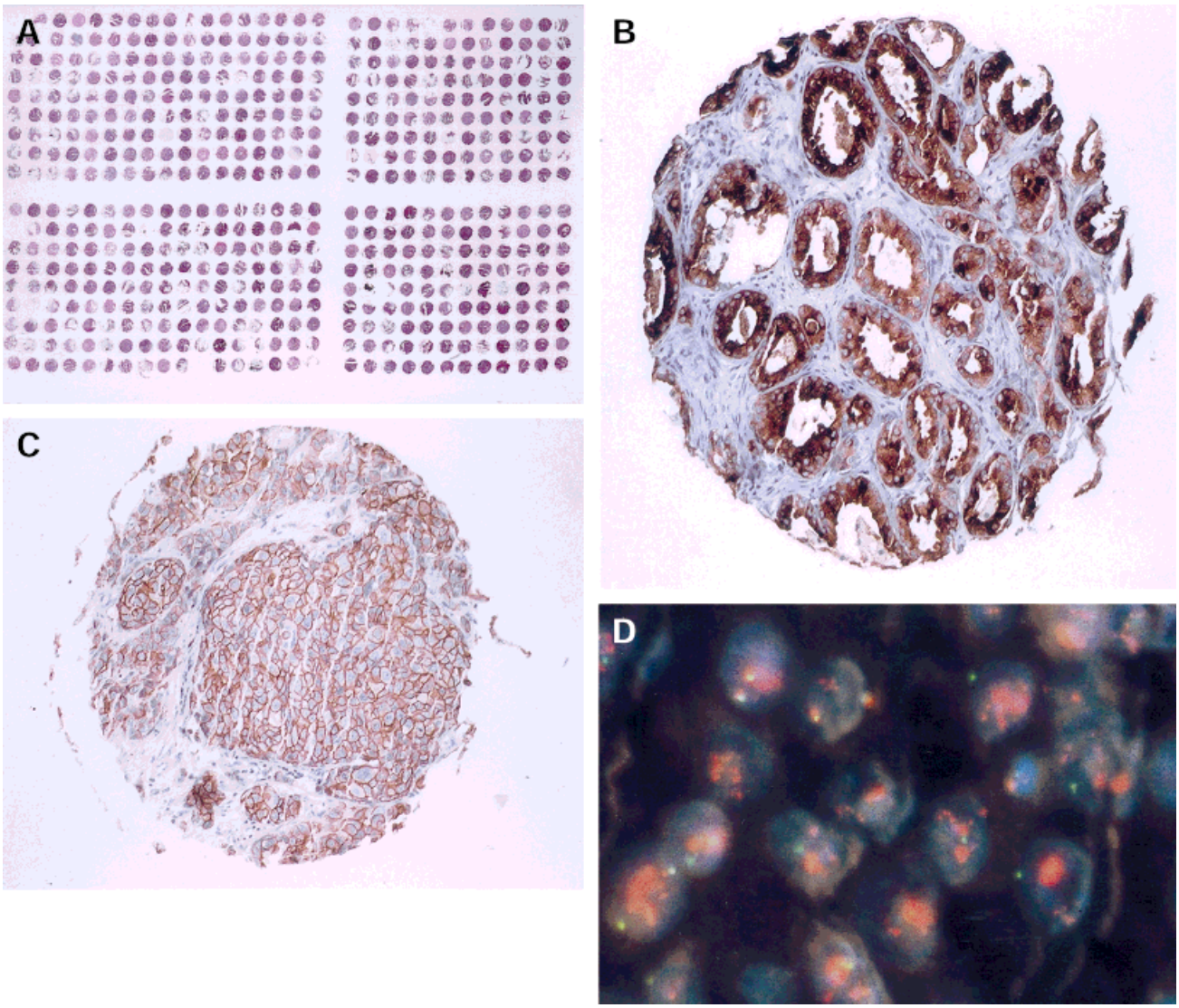

FIGURE 3 – Overview of different *in situ* analysis on tissue microarrays (TMAs). (*a*) HE-stained tissue-microarray slide. Specimens were arranged in 4 quadrants to facilitate orientation. Immunocytochemical detection of prostate-specific antigen (*b*) and herceptin (*c*) overexpression in arrayed tissue samples. (*d*) Cyclin D1-gene amplification detected by fluorescence *in situ* hybridization in 1 tissue-array element. All cells show a massive increase of their red cyclin D1 signals as compared with the green centromere cyclin D1 signals. Original magnifications ca. 5× for (*a*), ca. 100× for (*c*) and ca. 400× for (*d*).

371 prostate specimens,[11,12] consisting of 32 specimens of normal prostate or benign prostatic hyperplasia, 64 incidental carcinomas (stage pT1), 159 organ-confined carcinomas (pT2), 62 distant metastases and 54 recurrent tumors diagnosed after androgen deprivation treatment had failed. Molecular profiling of such tumor progression arrays revealed sets of genetic alterations and gene expression patterns that are characteristic of a specific stage of cancer progression.[11–13]

The strategy explained above for construction of tumor progression TMAs is based on using specimens from multiple patients. Although such arrays are highly informative as a population-level sampling tool, it is not possible to directly distinguish differences in gene expression caused by the cancer progression process from differences in gene expression between individual tumors/patients. To address this limitation, TMAs can also be constructed to study progression of cancer within the same tumor/patient. For example, specimens could be sampled into the same TMA from a primary tumor (including different histological patterns, various levels of differentiation within that tumor), adjacent normal tissue, preneoplastic lesions, local and distant metastases and from recurrent lesions obtained after treatment. At the Institute of Pathology in Basel we constructed a TMA composed of tissues from 196 nodal-positive breast carcinomas. From each patient, 1 sample was taken from the primary tumor and from each of 3 different metastases. Together with samples from 196 nodal-negative breast carcinomas, this "breast cancer metastasis TMA" contained almost 1,000 tissue samples. Such progression TMA will be highly informative in studying molecular alterations associated with metastatic progression of cancer.

"Patient outcome" TMAs

Patient outcome TMAs contain tumor samples from patients for whom clinical follow-up data are available, such as data on tumor recurrence, therapy response, time to metastasis or patient survival. These types of TMAs are most sensitive to the effects of sampling, which needs to be comprehensive enough to capture the clinically meaningful features of tumor aggressiveness. Analyses of known prognostic parameters (previously discovered and validated using

TABLE 1 – P53 IHC DATA OBTAINED FOR DIFFERENT GASTROINTESTINAL TUMORS IN A MULTITUMOR TMA

Tumor type	n	p53 positive tumors (%)
Oral cavity, normal tissue	4	0%
squamous cell carcinoma	17	42%
Salivary gland, normal tissue	6	0%
adenoma	8	0%
pleomorphic adenoma	34	0%
adenolymphoma	20	0%
adenoid cystic carcinoma	11	18%
acinus cell carcinoma	6	16%
other carcinoma types	15	13%
Pancreas, normal tissue	7	0%
adenocarcinoma	22	45%
Esophagus, normal tissue	9	0%
squamous cell carcinoma	16	43%
adenocarcinoma	3	100%
Stomach, normal tissue	10	0%
carcinoid	1	0%
adenocarcinoma	116	27%
Small intestine, normal tissue	9	0%
carcinoid	7	0%
adenocarcinoma	37	59%
Colon, normal tissue	6	0%
adenocarcinoma	309	59%
Colon adenoma, mild dysplasia	26	4%
moderate dysplasia	24	16%
severe dysplasia	24	16%
Appendix, normal tissue	6	0%
carcinoid	10	0%
Anus, normal tissue	2	0%
squamous cell carcinoma	3	0%
Gall bladder, normal tissue	8	0%
adenocarcinoma	13	30%
Liver, normal tissue	8	0%
Hepatocellular carcinoma	55	9%

conventional sections) almost invariably can be reproduced in TMA studies.[10,14] Studies executed in our laboratories have revealed the prognostic significance of estrogen receptor (ER), progesterone receptor (PR), *p53*, *HER2* (unpublished), and S6-kinase expression[15,16] in breast cancer, vimentin expression in kidney cancer[10] and cyclin E expression and Ki67 Labeling index in urinary bladder cancer.[8,9] In all cases in which previous studies were conducted on the same material with whole section sampling or when our data were compared with previous information in the literature, TMA analysis revealed a similar or even a better association with prognosis than large section studies. Although the result may vary depending on the type of the tumor or the particular biomarker of interest, current data suggest that TMAs will be ideally suitable for screening of the association of molecular markers with clinical endpoints. Before clinical application of such novel biomarkers, clinical correlations need to be validated based on regular whole section analysis.

EFFECT OF TUMOR HETEROGENEITY

The most commonly expressed concern is that TMAs acquire information from only a tiny area in each tumor. How can such a small site be representative of the entire tumor? Or alternatively, how many cores would need to be acquired to capture most of the information in an entire section? To address the influence of tumor heterogeneity and to evaluate the ability of TMAs to yield information on the prognostic value of biomarkers, 4 replicate TMAs were constructed from a series of 611 breast cancers.[17,18] The data will be described in detail elsewhere, but some of the main conclusions were: (*i*) The results from each replica array were almost identical, suggesting that the effects of intratumor heterogeneity are averaged out in such large-scale analyses; (*ii*) a single "punch" uncovered most of the information on ER expression in a tumor, whereas 4 punches were required in the case of PR; (*iii*) central and peripheral regions of the block gave identical results; and (*iv*) prognostic associations of the markers were always equally good or better when measured from the TMAs as compared with the analysis of individual large sections.

These data suggest that associations between molecular changes and clinical endpoints can be detected on TMAs, in many cases even based on a single core specimen per tumor. The data suggest that biomarkers that are of prognostic significance are more likely to be expressed in a relatively homogenous manner in the tumors.

Similar comparison studies between TMA analysis and conventional analysis need to be carried out with other tissues and other markers. Obviously, the more homogenously a marker of interest is expressed in the tumors and the more histologically homogenous the tumors are, the better the TMA data are likely to reproduce findings from whole section analysis. Comparing expression of 1 marker against another one on the same TMA, or comparison of marker expression between different stages of tumor progression included on the same microarray are less biased by the incomplete sampling of individual tumor specimens.

TMAs are not constructed from randomly or "blindly" selected regions of each tumor. Careful selection of the regions of interest in a morphologically heterogeneous tumor can substantially improve the representativity of the results. Some pathologists have been tempted to use larger-diameter punches (typically 2–4 mm) in an effort to increase the representativity of the TMA analysis. Although this approach multiplies the amount of tissue analyzed by a factor of 10 to 45, there is little mathematical or practical evidence that such a sampling strategy could be more representative than 0.6-mm punches. Typical primary tumors often measure 2–15 cm in diameter. Even if the concept holds that small subpopulations are critical for tumor behavior, the likelihood of detecting the decisive clone in a 3-mm sample is not much larger than detecting it in a 0.6-mm area. To improve the sampling efficiency, the acquisition of multiple small "tissue cores" from distinct, perhaps histologically different, regions of the tumor is dramatically more effective than increasing the size of a single punch. Based on these considerations we have selected the 0.6-mm sample size. Using larger (2–4-mm samples) also has the substantial disadvantage that it greatly reduces the number of samples that can be arrayed on a single slide (only about 50 4-mm biopsies fit on a single TMA slide) and the number of punches that can be taken from 1 original tumor block.

Finally, in judging the significance of the tissue microarray technology, one should keep in mind that tissue microarrays are a population-level screening tool to rapidly analyze frequencies of expression of biomarkers in cell populations, tissues or patients using large sample sets. They are not designed to characterize in detail every "corner" of a particular tumor mass. For this purpose, careful dissection of the tumor and consecutive sectioning throughout the tumor is needed.

FUTURE APPLICATIONS OF TMAS

Numerous possible applications of TMAs have not been fully explored. It is likely that this technology will be used in the future for transforming entire pathology laboratory archives into a "tissue chip" format. TMAs can be constructed not only from patient tissues, but also from cell lines and other experimental models such as xenograft tumors or tissues from transgenic animals. Combination of arrays from such experimental models with clinical TMAs will make it possible to transition seamlessly between experimental and clinical studies.

TMAs provide an ideal approach to compare different diagnostic or predictive markers or kits with one another on consecutive TMA sections. We performed such a study to compare *HER2* oncogene activation by immunohistochemistry and by FISH in breast cancer.[17] TMAs are also ideally suited for optimization and quality control purposes. Dozens of different staining or hybridization conditions can be tested (such as pre-treatments or tissues, probe/antibody labeling, detection, *etc*.) in replicate experiments to

achieve optimal performance of the assay. Appropriate positive and negative control tissues can be inserted into each TMA block as internal controls, and it is possible to measure variability of molecular detection schemes and determine which specific step in the detection process causes the most variability. All these issues will be critical to determine each new molecular marker to be introduced for diagnostic purposes. Finally, TMAs can be used for educational purposes. For example, trainees could compare their results on immunostaining scoring or tumor grading with those of a recognized expert.

Definitive clinical and prognostic studies that will be carried out in the future will be based increasingly on thousands of different tumors acquired from different institutions. TMA technology is ideally suited for acquiring tissues for molecular analyses from multiple institutions. Claims propagating the significance of a new diagnostic marker, probe or kit will be much stronger if the same results are obtained from multiple TMAs in different institutions. Although now only a few institutions have TMAs available for research, it is obvious that the availability of TMAs will become more widespread. The National Cancer Institute of the United States has launched an initiative to make TMAs available (http://resresources.nci.nih.gov/tarp/).

Finally, the technology of TMA construction and analysis is likely to improve. Automated array construction may soon increase the speed and quality of array making. Such increased throughput will rapidly begin to challenge the throughput of molecular pathology laboratories. For example, 1 technician could easily stain 40 TMA sections per day with a single antibody. If each section contained 500 specimens, almost half a million immunostaining results could be acquired in 1 month. Despite miniaturization, the reading and interpretation of these sections will be a significant task. This illustrates the critical need for automation of TMA analysis and interpretation in the future.

SUMMARY

TMAs will substantially accelerate studies associating novel molecular discoveries in the field of genomics and proteomics with specific pathologic, demographic, clinical and follow-up information of cancers and cancer patients. TMAs containing well-characterized tissues enable researchers to perform studies involving thousands of patient specimens with substantial increases in speed, quality of data, information content and savings of cost and time.

REFERENCES

1. DeRisi J, Penland L, Brown P, Bittner M, Meltzer P, Ray M, et al. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat Genet 1996;14:457–60.
2. Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM. Expression profiling using cDNA microarrays. Nat Genet 1999;21:10–4.
3. Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of hundreds of specimens. Nat Med 1998;4:844–7.
4. Battifora H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Lab Invest 1986;55:244–8.
5. Ayers LW. Application of the tissue microarray (TMA) method by the midregion AIDS and Cancer Specimen Bank (ACSB) to prepare study sets from HIV infected and control tissues. J Acquir Immune Defic Syndr 2000;23:A18.
6. Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Nocito A, et al. Tissue microarrays for gene amplification surveys in many different tumor types. Clin Cancer Res 1999;5:1966–75.
7. Tynninen O, Paetau A, Von Boguslawski K, Jaaskelainen J, Aronen HJ, Paavonen T. p53 expression in tissue microarray of primary and recurrent gliomas. Brain Pathol 2000;10:575–6.
8. Nocito A, Bubendorf L, Tinner EM, Süess K, Wagner U, Forster T, et al. High representativity of proliferation assessment and histologic grading on bladder cancer tissue microarrays. J Pathol, in press.
9. Richter J, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, et al. High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. Am J Pathol 2000;157:787–94.
10. Moch H, Schraml P, Bubendorf L, Mirlacher M, Kononen J, Gasser TC, et al. High-throughput tissue microarray analysis to evaluate the significance of genes uncovered by cDNA microarray screening in renal cell carcinoma. Am J Pathol 1999;154:981–6.
11. Bubendorf L, Kolmer M, Kononen J, Koivisto P, Mousses S, Chen Y, et al. Hormone therapy failure in human prostate cancer: analysis by complementary DNA and tissue microarrays. J Natl Cancer Inst 1999;91:1758–64.
12. Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser T, et al. Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence in situ hybridization on tissue microarrays. Cancer Res 1999;59:803–6.
13. Perrone EE, Theoharis C, Mucci NR, Hayasaka S, Taylor JM, Cooney KA, et al. Tissue microarray assessment of prostate cancer tumor proliferation in African-American and white men. J Natl Cancer Inst 2000;92:937–9.
14. Mucci NR, Akdas G, Manely S, Rubin MA. Neuroendocrine expression in metastatic prostate cancer: evaluation of high throughput tissue microarrays to detect heterogeneous protein expression [published erratum appears in Hum Pathol 2000;31:778]. Hum Pathol 2000;31:406–14.
15. Barlund M, Forozan F, Kononen J, Bubendorf L, Chen Y, Bittner ML, et al. Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. J Natl Cancer Inst 2000;92:1252–9.
16. Barlund M, Monni O, Kononen J, Cornelison R, Torhorst J, Sauter G, et al. Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. Cancer Res 2000;60:5340–4.
17. Bucher C, Torhorst J, Kononen J, Haas P, Askaa J, Godtfredsen SE, et al. Automated, high-throughput tissue microarray analysis for assessing the significance of HER-2 involvement in breast cancer [Abstract 2388]. Presented at the 36th Annual Meeting of the American Society of Clinical Oncology (ASCO); 2000 May 20–23; New Orleans, LA. Available at: http://www.asco.org/cgi-bin/prof/abst00.pl?absno=2388&div=sm&year=00abstracts.
18. Bucher C, Torhorst J, Bubendorf L, Schraml P, Kononen J, Moch H, et al. Tissue microarrays ("tissue chips") for high-throughput cancer genetics: linking molecular changes to clinical endpoints. Am J Hum Genet 1999;65(Suppl):A10.