Tissue microarray (TMA) applications: implications for molecular medicine

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Modern expression-screening platforms such as complementary DNA (cDNA) arrays allow for high-throughput lead discovery in cancer and other diseases. For evaluation of promising candidate genes, however, in situ analysis of high numbers of clinical tissues samples – for example, by immunohistochemistry or fluorescence in situ hybridisation – is mandatory. Tissue microarray (TMA) technology greatly facilitates such analysis. Minute tissue cores (diameter 0.6 mm) are removed from up to a thousand different conventional paraffin blocks and re-assembled in a single empty paraffin block at predefined positions. Sections of the resulting TMA can be utilised for the range of research applicable to conventional tissue sections. Important advantages of the TMA technology are speed (parallel analysis of up to a thousand tissues), cost efficiency (the same amount of reagents required for a single large-section analysis is sufficient for a thousand samples), and standardisation (the same experimental conditions are applied to all samples). Because of the high numbers of samples usually included in TMAs, they are optimally suited to detect genotype–phenotype associations with high statistical power. Thus, TMA technology will markedly accelerate the transition from basic research to clinical applications.

Recent advances in genome and transcriptome research have changed the landscape of biomedical research. DNA microarrays containing oligonucleotide or complementary DNA (cDNA) sequences representing thousands of genes allow the identification of genes or gene variants at the DNA and RNA level that are potentially disease related. As a consequence, hundreds of reports have been published on potentially interesting candidate genes from various neoplastic and non-

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neoplastic diseases, and the identification process continues apace. The upcoming branch of proteomics (defined as the systematic study of the complete complement of an organism's proteins) will further expand the quantity of diagnostic or therapeutic leads because different isoforms of one protein might exert different effects on the clinical course of disease.

The screening of the bulk of potential target genes or proteins in order to select those that have clinical importance is now a major challenge. Functional assays using in vitro or in vivo model systems, as well as the comprehensive analysis of large numbers of well-defined diseased and nondiseased tissues, are required to achieve this goal. However, high-throughput analysis of tissue samples using traditional methods has considerable disadvantages. In situ analyses such as immunohistochemistry (IHC), fluorescence in situ hybridisation (FISH) or RNA in situ hybridisation (RNA-ISH) can be cumbersome, slow and cost-intensive when using conventional methods of tissue sectioning. In addition, advances in surgical techniques increasingly allow for smaller biopsies, and precious tissue samples are quickly exhausted. Assays based on nucleic acid or protein extracts, such as polymerase chain reaction (PCR) analysis, northern or western blotting, and protein lysate arrays are well suited for high-throughput tissue analysis. However, they are based on the use of disintegrated tissues and, therefore, cannot provide the important information of which cell type or which cellular component in a tissue sample expresses the target gene.

To overcome these shortcomings, a tissue microarray (TMA) technique has been developed (Ref. 1). In this method, up to a thousand different, minute tissue samples can be placed onto one microscope glass slide and then be simultaneously analysed by in situ methods. The TMA technique has a number of advantages as compared with the 'sausage' block technique (Ref. 2) or the 'drinking straw' tissue core array that were introduced about 15 years ago (Ref. 3). Most importantly, the small diameter of the specimen taken out of the donor block, and the regular assembling in an array format with predefined coordinates for each tissue sample, maximises the number of samples that can be taken out of one donor block, minimises the tissue damage inflicted on it, and allows for automated analysis.

The TMA technology

The most important prerequisite for TMA construction is a large collection of well-characterised tissues – optimally with attached clinical data. About 95% of the workload of TMA construction is a histological and logistical exercise: after identification of potentially suitable cases from databases, hundreds to thousands (depending on the size of the planned TMA) of slides stained with haematoxylin and eosin have to be retrieved from the archive and subjected to careful histological review. Once all specimens have been selected that meet the histological requirements, the corresponding paraffin blocks are collected and sorted in the order of appearance in the TMA. In parallel, a database is generated providing the array coordinates for each tissue sample.

The arraying process itself is simple. Commercially available or homemade tissue microarray devices principally consist of three key components: (1) a hollow needle (typically about 0.6 mm in diameter), which is used to produce holes in an empty paraffin block (the 'recipient' block); (2) another hollow needle with a slightly thinner diameter to punch a tissue core from the tissue block (the 'donor' block) and to release the tissue core into the premade hole of the recipient block; and (3) a micromanipulated 'XY stage' that moves the recipient block to the predefined coordinates. Finally, a skilled person operating the microarrayer is required to complete the TMA.

The TMA can be cut like any paraffin block, using regular microtomes and equipment. However, an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA) facilitates the cutting. A tape is applied to the tissue block before sectioning in order to prevent damage to the TMA slice. The TMA section adhering to the tape is than transferred to an adhesive-coated glass slide, and the tape is removed. The tape system is particularly helpful in the case of large TMAs, which are more difficult to cut than small TMAs. The biggest advantage of the tape is that tissue loss during sectioning is minimised. This increases the number of sections that can be taken from each TMA block. TMA sections can be used for all types of in situ analyses, including IHC, FISH or RNA-ISH (Fig. 1).

TMA representativity

The most frequent concern about the TMA technology is whether the small size of the tissue

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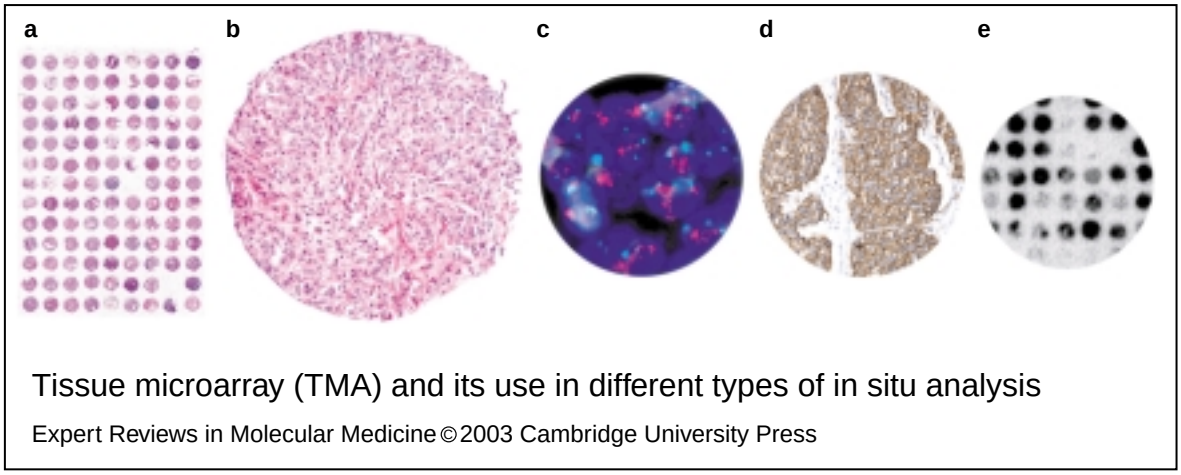

Figure 1. Tissue microarray (TMA) and its use in different types of in situ analysis. In principle, all kinds of research applicable to conventional tissue sections can also be carried out in a TMA format. (a) Haematoxylin and eosin (H&E)-stained section of a bladder cancer TMA containing 540 tissue spots. (b) Magnification of a single H&E-stained 0.6 mm tissue spot. (c) Fluorescence in situ hybridisation (FISH) analysis of centromere 17 (green signals) and the *HER2* gene (red spots) in cell nuclei (blue staining) of a tissue spot (magnification, 630×). (d) Immunohistochemistry (IHC) using an antibody directed against the HER2 protein shows strong brownish membranous immunostaining (magnification, 100×). (e) RNA in situ hybridisation (RNA-ISH) using a radioactively labelled oligonucleotide as a probe against β-actin mRNA; the black staining intensity indicates the level of mRNA in each tissue spot.

samples (0.6 mm) can be representative of an entire tumour (Refs 4, 5, 6, 7, 8). Obviously, small areas expressing a gene of interest can be missed and, especially in heterogeneous tumours, important features might not be present in the TMA sample. Approximately 20% of published studies on TMAs have addressed the issue of comparability of IHC results obtained from TMAs versus corresponding large sections (Refs 4, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28). In summary, the vast majority of these studies report a high concordance between the results obtained with the different approaches (Refs 4, 6, 7, 9, 10, 11, 13, 14, 15, 16, 17, 19, 20, 21, 23, 24, 26, 27, 28). Because the representativity of a TMA might be further improved if multiple tissue cores from the same donor tissue block are included, some investigators have performed experiments to determine the optimal number of tissue cores. It was found that two or three samples provided more-representative information than a single sample (Refs 4, 6, 19, 21, 24, 27) and that adding more than four or five samples had little effect on the concordance level (Refs 4, 19).

Camp et al. (Ref. 4) studied expression of the oestrogen receptor, progesterone receptor and *HER2/neu* oncogene product (all of which are common antigens in invasive breast carcinoma) in two to ten tissue cores obtained from the same donor blocks in a set of 38 invasive breast carcinomas. They found that analysis of two cores was sufficient to obtain identical results as compared with the corresponding whole-tissue sections in 95% of cases; 99% concordance was reached if four cores were analysed, and analysis of additional cores did not result in a significant further increase of concordance (Ref. 4). Similarly, Hoos et al. (Ref. 6) analysed one to three tissue cores from 59 fibroblastic tumours with heterogeneous Ki-67, p53 and retinoblastoma protein (pRb) expression. Analysis of three tissue cores yielded concordance rates of 91% (pRB), 96% (Ki-67) and 98% (p53) in comparison with the whole-tissue sections (Ref. 6). Recently, Rubin et al. (Ref. 19) analysed the Ki-67-labelling index (LI; fraction of cell nuclei positive for staining with the monoclonal antibody Ki-67) in ten separate cores of 88 prostate cancers. In this study, three cores were found to represent optimally the Ki-67 LI determined on a standard tumour slide. More than four cores did not add significant information (Ref. 19).

It is important to understand that all of these studies are based on the assumption that classical large sections – the current 'gold

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standard' for molecular tumour tissue analysis – are representative of an entire tumour. However, it is very well possible that this notion is not true. For example, a tumour with a diameter of 4 cm has a volume of approximately 33 cm^3. A 'large' section of this tumour, measuring 3 cm × 2 cm with a thickness of 3 µm, represents only 0.0018 cm^3. This makes up 1/18 300 of the whole tumour. A TMA sample measuring 0.6 mm in diameter represents 1/1600 of a 'large' section. In other words, although the potential lack of representativity between a TMA spot and the corresponding large section is an important concern, the problem is much greater between a large section and the corresponding tumour. These calculations suggest that, rather than comparing results obtained on large sections and on TMA sections, studies should determine whether or not established associations between molecular features and tumour phenotype or clinical outcome can be found in a TMA setting. Most importantly, one tissue core was enough to identify significant associations between molecular changes and previously well-established clinicopathological associations in all TMA studies that we are aware of (Refs 7, 14, 15, 27, 29, 30, 31, 32).

TMA applications

In principle, research that is applicable to conventional tissue sections can also be carried out in a TMA format. More than 130 studies using or reviewing the TMA technology have been published to date, the overwhelming majority of which are in the field of cancer research. Depending on the focus of the analysis, TMAs can be grouped into prevalence TMAs, progression TMAs, prognostic TMAs, and TMAs composed of experimental tissues.

Prevalence TMAs

Prevalence TMAs are 'simple' kinds of arrays containing tissue samples of one or more tumour type without any further pathological or clinical information. For instance, such TMAs are useful if the bare prevalence of a given alteration needs to be determined. For example, prevalence TMAs might contain a limited number (e.g. 20–100) of tissue samples from each of multiple different human tumour types. Such a 'multitumour' TMA is highly instrumental in analysis of the distribution and approximate expression frequency of a particular marker gene or protein across different cancer types. Similarly, TMAs from healthy tissues can be employed to describe comprehensively the tissue distribution of a particular marker.

In an early study, the known frequencies of amplification of the genes encoding cyclinD1, c-Myc and HER2 were confirmed in 17 different cancer types (Ref. 32). The largest 'multitumour' TMA manufactured so far in our laboratory contains 4788 different samples from 130 different tumour types and subtypes (Refs 33, 34). This TMA is currently utilised for the analysis of multiple different markers at the DNA and protein level. In one study, the frequency of cyclinE amplification and overexpression was analysed by FISH and IHC (Ref. 34). CyclinE was of particular interest as a significant association between cyclinE overexpression and poor prognosis was recently reported for breast cancer (Ref. 35). The multitumour TMA analysis detected cyclinE gene amplification in 15 different tumour types, including rhabdomyosarcoma, urinary bladder cancer, ovarian cancer, malignant fibrous histiocytoma, adenocarcinoma of the small intestine, medullary breast cancer, gall bladder adenocarcinoma, phaeochromocytoma, gastric adenocarcinoma, squamous cell carcinoma of the uterine cervix, colonic adenocarcinoma and endometrial carcinoma. CyclinE protein accumulation was found in 48 different tumour types, most frequently in Hodgkin's lymphoma (Ref. 34). This latter result is of particular interest from a technical point of view because Hodgkin's lymphomas predominantly consist of reactive inflammatory cells with only few dispersed neoplastic Hodgkin or Reed–Sternberg cells. It might therefore have been expected that TMAs would not be suitable for the study of Hodgkin's lymphoma. Other authors have also successfully used TMAs to analyse Hodgkin's lymphoma. For example, Hedvat et al. (Ref. 23) analysed a TMA containing Hodgkin's lymphomas for expression of CD20, CD30, CD15, Epstein–Barr virus (EBV) latent membrane protein 1 (LMP-1) and Epstein–Barr virus-encoded RNAs (EBERs) 1 and 2, and found the results matched exactly with those from whole sections corresponding to the tumour cores on the TMA.

Progression TMAs

Progression TMAs contain samples of different stages of one particular tumour type (Refs 1, 36, 37, 38). For example, an ideal breast cancer

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progression TMA would contain samples of (1) normal breast tissue from patients with and without breast cancer history, (2) several different non-neoplastic breast diseases, (3) ductal and lobular carcinoma in situ, and (4) invasive cancers of all stages, grades and histological subtypes, as well as metastases and recurrences after initially successful treatment. A multitude of studies have utilised progression TMAs to find associations between gene amplification or protein overexpression and tumour phenotype. Examples of relevant findings are shown in Table 1.

Prognostic TMAs

Prognostic TMAs contain samples from tumours with available clinical follow-up data. They are optimally suited to evaluate suspected associations between genetic alterations and clinical outcome of the patient. Several early studies using prognostic TMAs have confirmed the previously well-established associations between molecular findings and clinical outcome. Examples of relevant findings are shown in Table 2. In addition, molecular features have been analysed for their prognostic significance in cancer of the bladder (Refs 36, 39, 40), breast (Refs 11, 15, 31, 41, 42, 43), prostate (Refs 38, 44, 45), brain (Refs 20, 46, 47), liver (Ref. 48), kidney (Ref. 14) and colorectum (Refs 49, 50, 51), and in Hodgkin's lymphoma (Ref. 12) and malignant melanoma (Ref. 52).

TMAs from experimental tissues

TMAs can be made from all kinds of tissues. Paraffin-embedded cells harvested from cell cultures (Refs 40, 53) and xenograft tissues (Ref. 37) have been successfully analysed in a TMA format. A TMA containing a large number of different cell lines, for example, is a highly useful archive, allowing for time- and cost-efficient screening of hundreds of cell lines for a particular feature of interest.

Table 1. Examples of use of progression tissue microarrays (TMAs) to find associations between gene amplification or protein expression and tumour phenotype

Gene or protein alteration	Associated tumour phenotype	Ref.
CyclinE gene amplification	Bladder cancer stage and grade	56
FGFR1 or *RAF1* gene amplification		57
MDM2 or *CDK4* gene amplification		40
MAGE-A4 protein expression		58
Cytokeratin 7 and 20 protein expression	Colorectal cancer grade	59
IGFBP2 protein overexpression	Prostate cancer hormone-refractory state	37
EIF3S3 gene amplification	Prostate cancer stage	60
Aneusomy of chromosomes 7, 8 and 17		61
Loss of E-cadherin protein expression	Prostate cancer tumour size	62
E-cadherin protein expression	Brain tumour aneusomy and type	63
SHP1 expression	Lymphoma development	64
Combined loss of PTEN and p27 expression	Prostate cancer recurrence	65

Abbreviations: CDK4, cyclin-dependent kinase 4; EIF3S3, p40 subunit of eukaryotic translation initiation factor 3 (eIF3); FGFR1, fibroblast growth factor receptor 1; IGFBP2, insulin-like-growth-factor-binding protein 2; MAGE-A4, melanoma antigen family A4; MDM2, mouse double minute 2 homologue; p27, cyclin-dependent kinase inhibitor 1B; PTEN, phosphatase and tensin homologue; RAF1, v-*raf*-1 murine leukaemia viral oncogene product homologue 1; SHP1, *Homo sapiens* nuclear hormone receptor.

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Table 2. Examples of use of prognostic tissue microarrays (TMAs) to find associations between gene amplification or protein expression and clinical outcome

Gene or protein alteration	Associated clinical outcome	Refs
Oestrogen/progesterone receptor protein expression	Shortened patient survival in breast cancer	15
HER2 gene/protein alterations		31
Vimentin protein expression	Shortened patient survival in kidney cancer	14
Ki-67 labelling index[a]	Shortened patient survival in urinary bladder cancer	7
Ki-67 labelling index	Shortened patient survival in soft tissue sarcomas	29
Ki-67 labelling index	Shortened patient survival in Hurthle cell carcinoma	30
17q23 genomic amplifications	Shortened patient survival in breast cancer	31
COX2 protein expression		41
TOP2A protein expression	Shortened patient survival in glioblastoma	46
MYC and AIB1 protein expression	Shortened patient survival in hepatocellular carcinoma	48
IGFBP2 protein expression	Shortened patient survival in prostate cancer	37

[a] Ki-67 labelling index is the fraction of cell nuclei positive for staining with the monoclonal antibody Ki-67.
Abbreviations: AIB1, nuclear receptor coactivator 3; COX2, cyclooxygenase 2; HER2, avian erythroblastic leukaemia viral (v-*erb*-b2) oncogene product homologue 2; IGFBP2, insulin-like-growth-factor-binding protein 2; MYC, avian myelocytomatosis viral (v-*myc*) oncogene product homologue; TOP2A, topoisomerase 2 alpha.

Automation

Automation is a key issue for all work-intensive tasks, and automated TMA devices have recently become available. With respect to TMAs, two steps are particularly suited for automation: the arraying process itself and the interpretation of stained TMA slides. Automated TMA devices have the advantage of yielding a more-regular appearance to the TMAs, although similar improvement of the TMA quality can also be achieved by minor homemade adjustments to existing manual arrayers. By contrast, the interpretation of the stainings constitutes a much more significant bottleneck in high-throughput operations. For example, projects aiming at the comprehensive analysis of several thousands of tumours with a multitude of different markers only become feasible using the TMA technology. Such projects can easily lead to millions of tissue spots that must be evaluated. Well-trained pathologists are able to interpret approximately a thousand tissues per hour. Even if this work speed could be maintained for 8 h per working day, no more than 40 000 tissue spots could be analysed per week. Consequently, about four months of continuous work would be required to score a million tissue spots. Considering these time constraints, it becomes evident that automated TMA analysis is required for large-scale TMA studies. In particular, TMAs are

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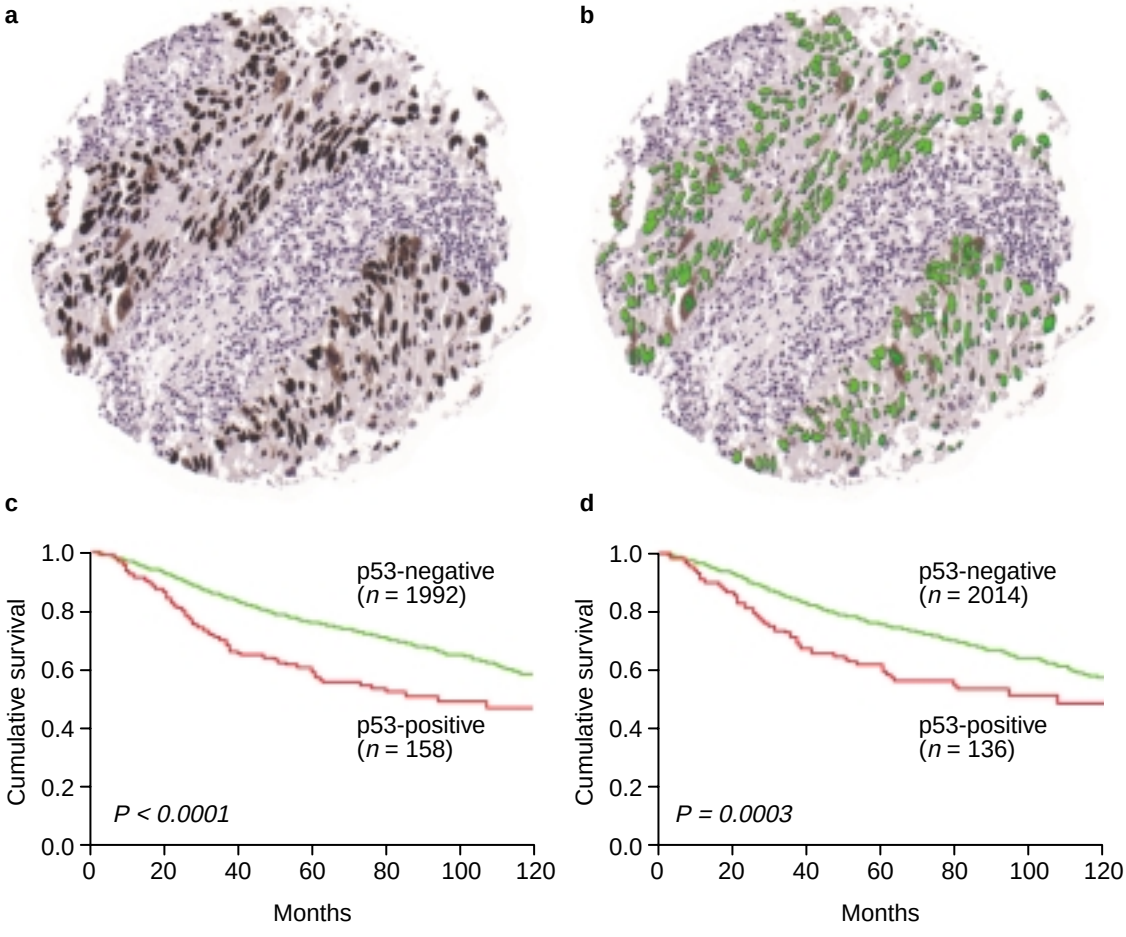
optimally suited for automated IHC analysis. The most crucial step for automation of IHC analysis is the selection of the area to be analysed; this selection has already been made in TMAs.

Automated scoring of TMAs requires specific equipment. First, a fully automated and computer-controlled microscope is necessary, optimally with slide-changing capabilities that allow stacking of multiple TMA slides for continuous analysis. Second, 'intelligent' software is needed to locate the spots on the slide, capture images of every spot, link the images to a database and measure the staining intensity. In principle, these requirements are fulfilled if features of histological image analysis software and array analysis software (e.g. as it is used for cDNA array analysis) are combined. For this purpose, the use of conventional peroxidase-based immunostaining as used for conventional bright-field analysis is desirable because the same



Tissue microarray (TMA) analysis: manual versus automated

Figure 2. Tissue microarray (TMA) analysis: manual versus automated. (a) Immunohistochemistry showing p53 expression (brown nuclear staining) in a breast cancer tissue spot. (b) Same image as in (a), with green colour indicating automated thresholding for positive p53 staining. Survival plots were calculated from both manual (c) and automated (d) analysis: both plots indicated a significantly worse prognosis for patients with immunohistochemically detectable p53 expression [M. Ramseier, S. Hänggi and J. Wirth (Institute of Pathology, University of Basel, Switzerland), pers. commun.]. Abbreviations: *n*, number of samples analysed; *P*, chi square *P* value.

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slides can be used for conventional 'manual' interpretation and for automated analysis. Our analysis of multiple markers with a simple homemade automated system revealed the expected associations with outcome information in all analysed cases. As an example, the results obtained for manual and automated p53 staining analysis in breast cancer are shown in Figure 2. However, despite the similarity in results from manual versus automated scoring, such a rough approach has several evident weaknesses. The lack of a distinction of tumour cells from non-neoplastic cells is probably the most important issue in this respect. There are genes, such as calretinin, that are sometimes expressed in cancer cells and sometimes in the stroma cells of tumours (Ref. 54). A purely intensity-based measurement without tumour cell recognition is not suitable in this case. One recent study by Camp et al. (Ref. 55) has shown that sophisticated automated analysis is feasible. However, demanding multicolour fluorescent IHC was needed for this purpose.

Future directions

TMA analysis has become a widely used, standard technology. To date, TMAs are available from nonprofit organisations such as academic departments or TMA facilities (e.g. the National Human Genome Research Institute, http://research.nhgri.nih.gov/microarray/main.html), and a small number of commercial sources. Commercial TMAs are typically small and have few clinical data attached. However, the number of institutions having TMA manufacturing facilities is increasing rapidly. It is anticipated that virtually all institutions dealing with tissue-based research (e.g. pathology institutes) will be using TMAs in the future. Thus, it is expected that the number of commercial and academic TMA providers will also increase. Along with an increasing availability of TMAs, more emphasis will be put on the development of automated systems for TMA analysis. The implementation of web-based database resources will allow the combined online analysis of all data obtained from one TMA, even from different investigators. This will be particularly important as multiple 'identical' replicate TMAs can be manufactured from one set of well-defined tissues if multiple punches are taken from a donor block and distributed into separate recipient blocks. Assuming that 100 replicate arrays can be made from a medium-sized (1.5 cm^2) piece of donor tissue, and approximately 200 sections can be cut from each of the arrays, the expression status of 20 000 different genes (which is about half of the human genome) could be analysed and subjected to cluster analysis with unprecedented statistical significance. Because of the tissue-saving aspect, TMAs should also play a key role in the analysis of tissue samples from clinical trials. These samples are usually small and precious as they contain homogeneously treated tumours, and would not be unnecessarily exhausted by material-consumptive analyses.

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Further reading, resources and contacts

A summary (including a slide show) of the National Human Genome Research Insitute's Tissue Microarray Project can be found at:

http://research.nhgri.nih.gov/microarray/main.html

Manufacturers of Tissue Arrayers:

Chemicon (http://www.chemicon.com/company/PR/Arrayer.asp)
Beecher Instruments (http://www.beecherinstruments.com/)

TMA sectioning aid system:

Instrumedics Inc. (http://www.instrumedics.com/)

Automated TMA analysis systems:

Chromavision (http://www.chromavision.com/prod/microtissue/index.htm)
TissueInformatics (http://www.tissueinformatics.com/products/array.html)

Features associated with this article

Figures

Figure 1. Tissue microarray (TMA) and its use in different types of in situ analysis.
Figure 2. Tissue microarray (TMA) analysis: manual versus automated.

Tables

Table 1. Examples of use of progression tissue microarrays (TMAs) to find associations between gene amplification or protein expression and tumour phenotype.
Table 2. Examples of use of prognostic tissue microarrays (TMAs) to find associations between gene amplification or protein expression and clinical outcome.

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