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Review Article

Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput *in situ* studies

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Abstract

Tissue microarray (TMA) technology allows a massive acceleration of studies correlating molecular *in situ* findings with clinico-pathological information. In this technique, cylindrical tissue samples are taken from up to 1000 different archival tissue blocks and subsequently placed into one empty 'recipient' paraffin block. Sections from TMA blocks can be used for all different types of *in situ* tissue analyses including immunohistochemistry and *in situ* hybridization. Multiple studies have demonstrated that findings obtained on TMAs are highly representative of their donor tissues, despite the small size of the individual specimens (diameter 0.6 mm). It is anticipated that TMAs will soon become a widely used tool for all types of tissue-based research. The availability of TMAs containing highly characterized tissues will enable every researcher to perform studies involving thousands of tumours rapidly. Therefore, TMAs will lead to a significant acceleration of the transition of basic research findings into clinical applications.

Keywords: tissue microassays; high-throughput technologies; immunohistochemistry; FISH; *in situ* hybridization

Introduction

A draft sequence of the whole human genome has recently been completed and a gene map containing the locations of the first 30 000 genes can be accessed on the internet (http://www.ncbi.nlm.nih.gov/gene map). Detailed information on the sequence of genes has set the stage for the development of highly powerful expression screening tools. High-density cDNA microarrays enable the simultaneous analysis of the expression levels of thousands of genes. This technology allows the monitoring of the gene expression status of a tissue on a global basis and will rapidly highlight genes and signalling pathways that are involved in neoplastic and non-neoplastic diseases [1,2].

With the availability of such powerful approaches, the next challenge will be to apply the genome discoveries in the clinical setting, such as diagnostic and prognostic assessment, therapy target identification, individualized therapy, and preventive strategies. An especially high number of potentially significant genes are expected in the cancer field. It is well known that alterations of multiple different genes in each of multiple different pathways can lead to cancers that may look identical by standard microscopy. As the rate of discovery of new genes involved in cancer initiation and progression increases, a rapidly growing demand for analyses of these new genes on primary tumours can be expected. To identify the clinically most significant alterations among all the emerging candidate genes, it will soon be necessary to analyse a large number of genes in large sets of well-characterized tumours. Ideal tissue collections would include tumours of all stages, precursor lesions, metastatic tissue, cancers with known clinical outcome or defined response to therapy, as well as normal tissues.

Such studies require the analysis of hundreds of tumours to provide statistically meaningful results. Therefore, the investigation of a large number of different candidate genes leads to a massive workload in the involved laboratories. Moreover, the analysis of multiple genes results in a critical loss of precious tissue material, since the number of conventional tissue sections that can be taken from a tumour block does not usually exceed 200–300.

Tissue microarrays (TMAs)

Our recently developed tissue microarray (TMA) technology significantly facilitates and accelerates tissue analyses by *in situ* technologies [3]. In this method, minute tissue cylinders (diameter 0.6 mm) are removed from hundreds of different primary tumour blocks and subsequently brought into one empty 'recipient' paraffin block (Figure 1). Sections from such array blocks can then be used for simultaneous *in situ* analysis of hundreds or thousands of primary tumours on the DNA, protein, and also RNA level. A related technology, termed the multi-tissue or 'sausage' block technique, was introduced more than 10 years ago [4]. In this method, larger fragments are taken from donor blocks and then brought into a new

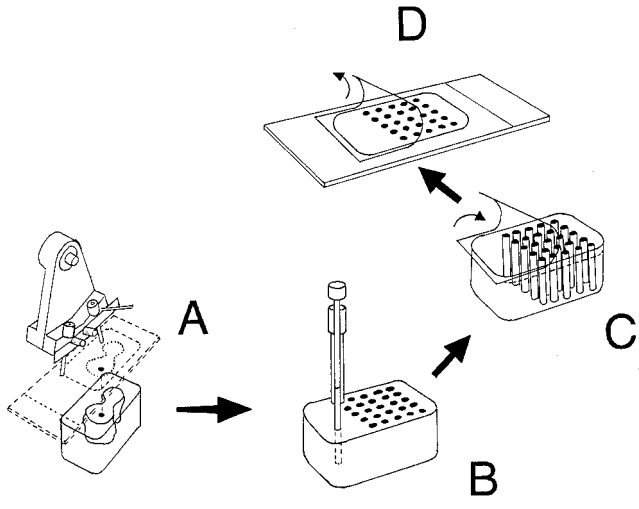

Figure 1. TMA construction. (A) A tissue core biopsy of 0.6 mm in diameter is punched from a preselected region of a donor block using a thin-wall stainless steel tube. A haematokylin and eosin (H&E) stained section overlaid on the surface of the donor block is used to guide sampling from representative sites in the tissue. (B) The tissue core is transferred into a premade hole at defined array coordinates in the recipient block. (C, D) An adhesive-coated tape sectioning system assists in cutting the tissue microarray block

recipient block in a less organized way than in TMAs. Although related concepts have been used, the TMA technique has substantial advantages over the sausage block technique. For example, the cylindrical form and the small diameter of the specimen taken out of the donor block maximize the number of samples that can be taken from one donor block and minimizes the tissue damage inferred to the donor block. The latter is important for pathologists, since they can now give researchers access to their material and at the same time, retain virtually undamaged tissue blocks. Punched tissue blocks remain fully interpretable for all morphological and molecular analyses that may subsequently become necessary, if a reasonable number of punches is selected. Figure 2 illustrates that dozens of punches can be taken from one tumour without compromising interpretability. If a tissue block can be exhausted, more than 300 punches can theoretically be collected from a tumour block containing a tumour area of 18×18 mm. Since the entire thickness of the donor block tissue is represented in each of the cylindrical tissues of a TMA block, an equal number of sections can be taken from TMAs, as it would have been possible to cut from the corresponding donor blocks. Therefore, it would be possible to perform about 90 000 *in situ* analyses (300×300) from one set of tumours if every sample had a diameter of ≥ 18 mm. Another distinct advantage of TMAs is the exact X–Y positioning of each specimen. The precise arrangement of arrayed samples not only facilitates manual interpretation of the staining, but also serves as an ideal basis for automated analysis.

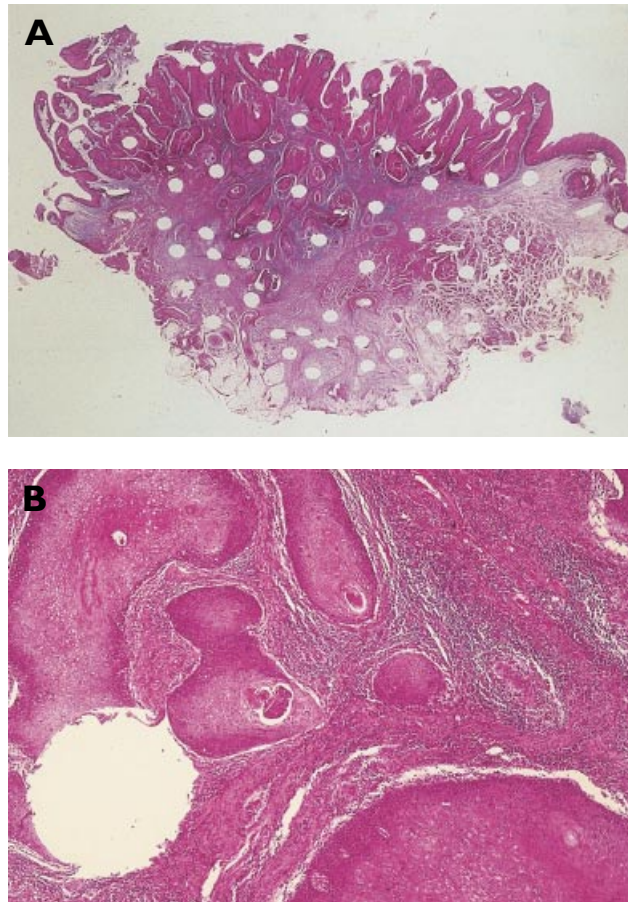

Figure 2. Effect of multiple punches on donor blocks. More than 50 punches have been taken from this block of a tonsil carcinoma. As can be seen at low (A; $\times 3.25$) and even better at higher (B, $\times 39$) magnification, tissue interpretability does not suffer significantly from the procedure

TMA construction

Although a device is needed to manufacture TMAs, it cannot be emphasized enough that most of the work (approximately 90%) involved in making TMAs is traditional pathology work that cannot be accelerated by improved tissue arrayers. This preparatory work is very similar to what is needed for traditional studies involving 'large' tissue sections. The major difference may lie in the number of tissues involved, which is typically an order of magnitude higher in TMA studies than in traditional projects. First, a list of potentially suited tissues needs to be generated. Then all sections of these candidate specimens are collected from the archive and reviewed by a pathologist in order to select optimal donor blocks. During this process, the donor tissues can be reclassified according to current classification schemes and tissue areas suited for subsequent punching can be marked. Different colours are recommended for marking different areas on one section. For example, we always use red for tumour, black for carcinoma *in situ*, and blue for normal tissue in our tumour studies. Subsequently, the selected blocks are collected from the archive. These blocks

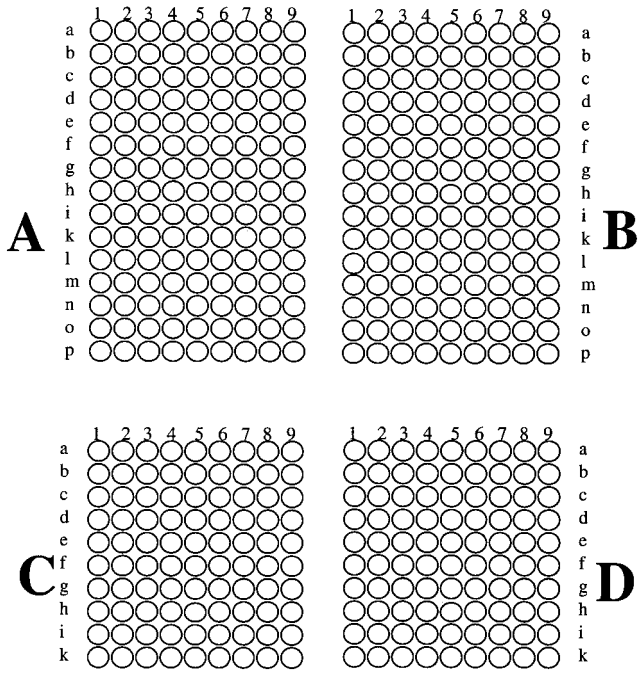

Figure 3. Diagram of TMA organization. Tissue samples are distributed in multiple divisions

and their corresponding marked slides must then be brought together and sorted in the order in which they should appear on the TMA. At the same time, the outline of the TMA needs to be defined (Figure 3) and a file should be generated that contains the identification numbers of the tissues together with their locations and the coordinates to be selected on the arrayer. To facilitate orientation on the TMA, arranging the tissues in multiple sections (e.g. quadrants) is strongly recommended. In most laboratories, capital letters define these quadrants, while lower-case letters and numbers define the coordinates within these quadrants. Examples of a data file containing the necessary information for making a TMA are given in Table 1.

Only if all of this preparatory work has been done can a tissue arraying device be employed. The only tissue arraying system that is commercially available is identical to the prototype machine used in the early development of TMA technology (Beecher Instruments, Silver Springs, MD, USA). Using this manually operated device, excellent TMAs can be produced in the hands of a talented and experienced person. However, this cannot be expected before a significant training period, mostly including several hundred, if not a few thousand, punches. A patient and enduring personality as well as good eyes are important prerequisites for operators of the current manual tissue arrayers. Although it is possible to take regular microtome sections from a TMA block, a tape sectioning kit (Instrumedics Inc., NJ, USA) is recommended for sectioning. This efficiently prevents arrayed samples from floating off the slide if harsh pretreatments are used in the course of *in situ* analysis protocols.

Table 1. Example of a TMA data file. Note that once the TMA is constructed, permanent unlinking of research and patient data can be achieved by destroying the column containing the patient identifier

Biopsy No.	Localization	Coordinates	pT	pN	Grade
B85.45064	A1a	0/0	2	1	1
B85.45020	A1b	800/0	4	1	1
B85.45366	A1c	1600/0	2	1	1
B85.45855	A1d	2400/0	3	1	2

Tissue heterogeneity

The most obvious question linked to TMA technology is to what extent tumour heterogeneity would affect the validity of the TMA approach. These concerns were extensively addressed in a series of early TMA studies. From these results, it could be concluded that all findings that had previously been found by *in situ* methods on large sections, or by other methods on large tissue samples, could be fully reproduced in TMA studies. Some key studies providing evidence for the validity and representativity of TMA data are summarized below. Kononen *et al.* [3] found the same frequencies of HER-2, c-myc, cyclin D1 and 17q23 amplifications in breast cancer as were expected from previous data of the literature. In the same study, known associations between tumour phenotype and gene amplifications were confirmed. For example, HER-2 amplification was significantly associated with high-grade tumours. Nocito *et al.* (A) examined the histological grade and the Ki67 labelling index (LI) on four replica TMAs, each containing samples from 2317 different bladder carcinomas. In this study, Ki67 LI was also determined on large sections in a subset of tumours. All associations of the histological grade or Ki67 LI with prognostic information or tumour phenotype that were found in large section analysis could be fully reproduced on all four replica TMAs. Torhorst *et al.* (manuscript submitted) generated a breast cancer array including 553 cancers with survival information. Again, four replica arrays containing different samples from each tumour were constructed. The results showed that the well-established prognostic significance of oestrogen receptor (ER) and progesterone receptor (PR) positivity could be entirely reproduced on every individual TMA, independently of whether the samples were taken from the centre or the periphery of the tumours. The same breast cancer prognosis array was also utilized to confirm the known prognostic significance of HER-2 amplification and overexpression (C. Bucher, unpublished data). Schraml *et al.* [5] constructed a multi-tumour array containing 397 samples from 17 different tumour types. This array was used to see whether the known amplification frequency of HER-2, MYC, and CCND1 (genes that

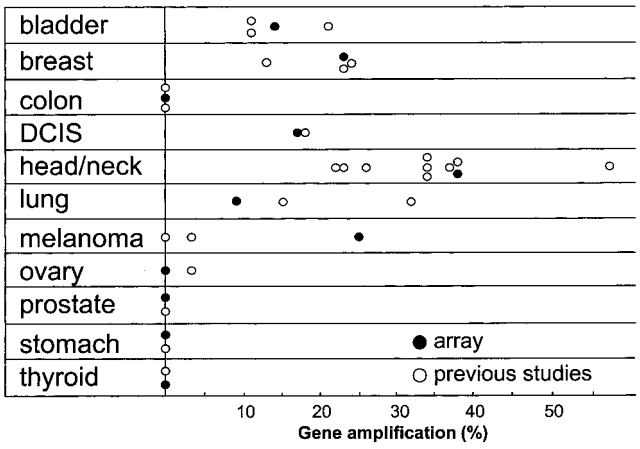

Figure 4. Cyclin D1 amplification analysis of a multi-tumor TMA. The frequency of cyclin D1 amplification as determined on a TMA (closed circles) is very similar to previous data obtained by other methods (open circles)

were extensively analysed in previous studies) could be reproduced on a TMA. The results showed impressive concordance between the TMA data and the previous literature. Figure 4 shows the results obtained for CCND1 in comparison with the previous data. In fact, the results of this study suggested that three hybridizations, executed and analysed within less than 2 weeks, allowed most of the knowledge to be reproduced that had previously been accumulated through more than 100 different studies including more than 8000 different experiments.

To understand the apparent lack of a major influence of tissue heterogeneity on the results of TMA analyses, it is important to realize that the tissue array approach has been designed to survey tumour populations and not to examine individual tumours. It is unavoidable that some alterations are not detected if the analysis of potentially heterogeneous tumours is restricted to samples measuring 0.6 mm in diameter. The inability to detect all alterations on a TMA is not only due to true heterogeneity, but also to technical problems often preventing reliable analysis in some areas of a fixed tumour. However, it can be assumed that the likelihood of error will be similar in all tumour groups represented on one array. Associations between molecular alterations and clinical or morphological parameters (stage, grade, prognosis) are therefore likely to be similarly detectable on sufficiently large TMAs as in large section analyses, although the absolute frequency of a given alteration may be an underestimate of its true prevalence.

A frequent question arising in the context of tissue heterogeneity is whether a larger diameter of the needle used for TMA construction (for example, 3 mm instead of 0.6 mm) could help to minimize sampling errors caused by heterogeneity. Although we started the development of TMA technology using 3 mm needles, we have not performed specific experiments to address the influence of needle diameter on the detection of heterogeneous tumour markers. It is felt, however, that increasing the needle size will be of very limited value for this purpose, since tumour heterogeneity is relatively unlikely to be detected within a still very small tissue fragment measuring 3 mm in diameter. If a better representation of an individual tumour is requested, it will be much more advantageous to array two or more samples from different areas of each tumour.

TMA staining and analysis

TMAs are suited for all analyses that can be done *in situ*. This includes immunohistochemistry, fluorescence *in situ* hybridization (FISH), and RNA *in situ* hybridization (RNA-ISH). In general, the same protocols can be used as for large sections. Examples of stained TMA sections are shown in Figure 5. The most significant difference compared with traditional large section studies lies in the unprecedented level of standardization that can be achieved within one TMA experiment. All the slides of one TMA study are usually incubated within one jar, ensuring absolutely identical concentrations and temperatures of all reagents. Moreover, the age of a slide (time between sectioning and use), section thickness, and the exposure times for all steps are also exactly identical in these studies, where all the tissues of one study are located on the same TMA slide. This is a significant difference compared with traditional studies, where a slight variability in some minor experimental parameters (such as slide age or section thickness) is perceived as tolerable. As a result of extreme standardization within each experiment, surprising intra-laboratory variations can sometimes occur if experiments are repeated under slightly different conditions. Importantly, these variations affect the overall frequency of positivity but they do not seem to involve associations between the examined parameters and clinico-pathological data. This is obviously because all groups within one TMA (low and high stage, good and poor prognosis) are equally affected by experimental variations and significant differences will still become detectable if varied experimental conditions alter the threshold for the detection of positivity. Obviously, a sufficiently large number of tissues on a TMA are important to find significant associations in the case of sub-optimal immunostaining.

If hundreds of biopsies were all treated individually at the time that they were removed, fixed in buffered formalin, and subsequently paraffin-embedded, it must be expected that these tissues will be heterogeneous in their preservation of proteins and nuclear acids. That this is true is best illustrated in the outcome of FISH analyses. As on large sections, TMA FISH analyses provide interpretable results in only about 60–90% of analysed tumours at the first attempt. As in large section studies, it is possible to achieve the interpretability of a fraction of initially non-informative cases by changing the experimental conditions. For example,

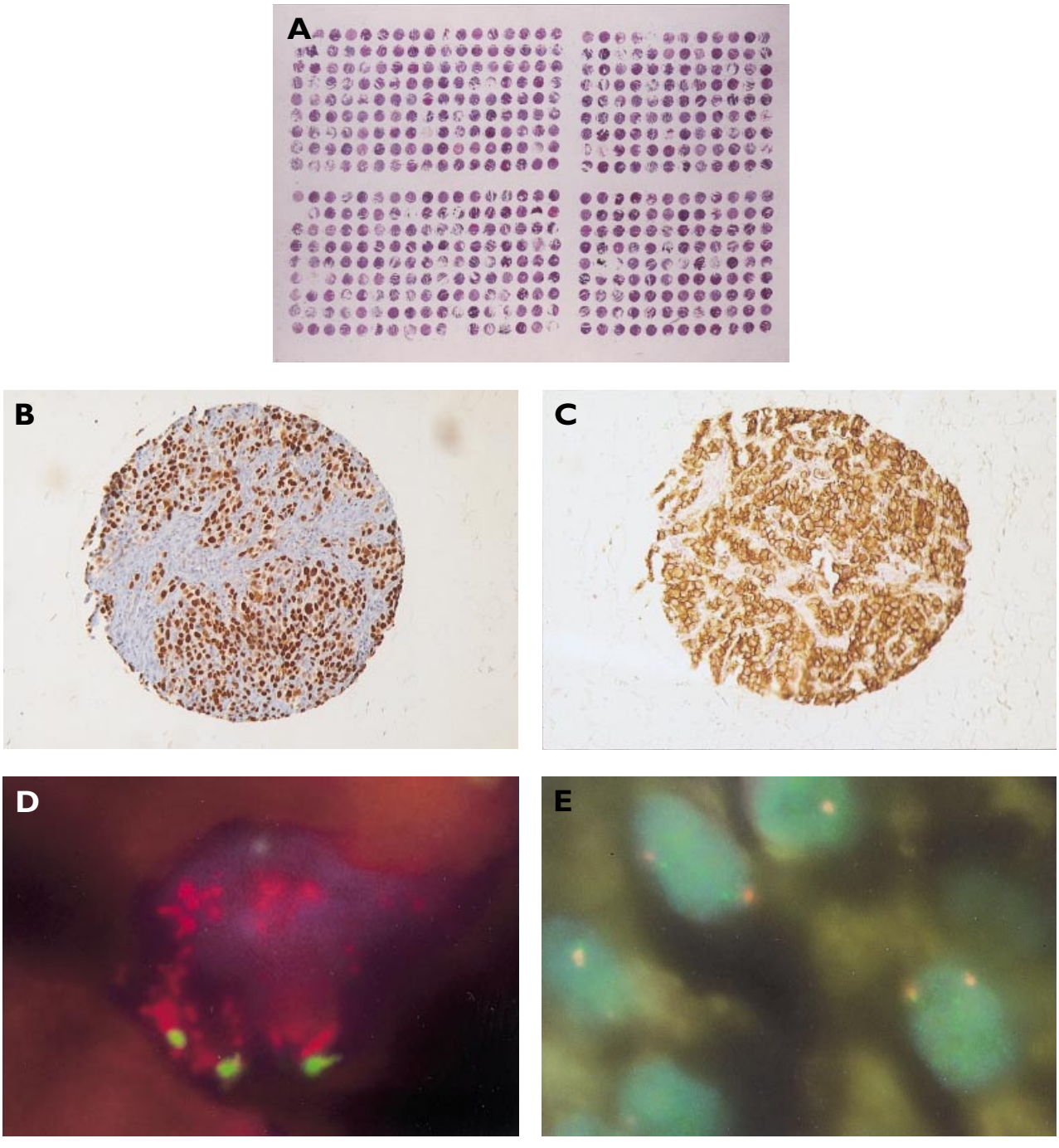

Figure 5. Examples of TMA staining. (A) Overview of a TMA slide (H&E). (B) p53-positive tumour sample. (C) HER-2-positive tumour sample. (D) Cells from a tumour with HER-2 amplification showing a massive increase of the red labelled HER-2 genes (>25) but only three green centromere 17 signals. (E) Tumour cells with normal centromere 17 (green signals) and HER-2 gene (red signals) content

an increased proteinase K concentration in the slide pretreatment will result in interpretable signals in some initially non-informative cases at the cost of over-digestion of some previously interpretable samples. In general, we do not attempt to achieve a higher fraction of FISH-informative cases by changing the experimental conditions. Because of the large number of tumours on most TMAs (usually >500), we tolerate a significant fraction of non-interpretable tumours rather than using too many precious TMA sections for additional experiments. The question of whether RNA-ISH analysis can be done reliably on sections from archival tissue is highly disputed. Laboratories that feel confident in performing RNA-ISH analyses on formalin-fixed sections will also be able to execute such analyses on TMAs.

TMA applications to study tumour biology

Virtually all tissues are suitable to be placed into a TMA. Therefore, the range of TMA application is as

broad as the imagination of the users of this technology. One of the most distinct advantages of TMAs is the fact that one set of tissues that has been reviewed once by a pathologist and for which clinical data have been collected can now be used for an almost unlimited number of studies. The TMA technique is not at all limited to cancer research. It is a matter of time until valuable TMAs will be made for other research areas where examinations are needed, such as in inflammatory, cardiovascular or neurological diseases. Nevertheless, TMAs have so far mainly been used in cancer research. Typical TMAs that have been constructed include multi-tumour, progression, and prognostic arrays.

Multi-tumour TMAs are composed of samples from multiple tumour types. These arrays are utilized to screen different tumour types for molecular alterations of interest. For example, the largest multi-tumour TMA constructed by our groups contains 4788 samples (distributed among ten TMA blocks) of 130 tumour types, including also 354 normal tissues and 709 metastatic cancers. Sections from such arrays can be utilized to analyse comprehensively the role of newly identified genes in normal and neoplastic human tissues.

Progression TMAs have been used to study molecular alterations in the different stages of one particular tumour type [3,6–8]. For example, an ideal prostate cancer progression TMA would contain samples from either normal prostate or benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), incidental carcinomas (stage pT1), organ-confined carcinomas (pT2), carcinomas with extraprostatic growth (pT3–4), as well as metastases and recurrences after androgen withdrawal treatment. Such a prostate cancer TMA would also contain tumours of all the different Gleason grades. Importantly, the Gleason pattern, which varies within one prostate cancer, can be defined for the very region that was included in the TMA. As a consequence, the prostate cancer TMA data sheet would contain not only the primary and the secondary Gleason pattern, but also the 'arrayed Gleason pattern'. TMAs are also suited to study progression within tumours. TMAs can easily include large numbers of pairs of primary tumours and their non-invasive precursor lesions, metastases, or recurrences after specific treatment. In our laboratory, we have constructed a TMA composed of tissues from 196 nodal-positive breast carcinomas. From each tumour, one sample was taken from the primary tumour and of each of three different metastases. Together with samples from 196 nodal-negative breast carcinomas, this 'breast cancer metastasis TMA' contains almost 1000 tissue samples. In a recent study, Simon *et al.* (unpublished data) used this array to show that high concordance in the HER-2 amplification pattern exists between primary tumours and their nodal metastases.

Prognosis TMAs contain samples from tumours with available clinical follow-up data. In multiple studies, we have used prognosis TMAs to examine molecular alterations that were known to have prognostic significance in tumours. Associations with prognosis were found for ER, PR, p53 positivity and HER2 amplification/overexpression in breast cancer (J. Torhorst and C. Bucher, unpublished data), cyclin E expression [6] and Ki67 LI in urinary bladder cancer (A), and vimentin expression in kidney cancer [9]. These data demonstrate that associations between molecular changes and clinical end-points can be detected on TMAs containing just one single specimen per tumour, especially if the number of tumours included in a TMA is large. Prognosis TMAs were also used to analyse novel genes. For example, amplification of S6 kinase was found to be associated with poor prognosis in breast cancer [10].

Other TMA applications are obvious but have not yet been largely exploited. For example, testing the expression pattern of gene products can optimally be done on TMAs containing all kinds of normal tissues. Similarly as for patient tissues, TMAs can be used for cell lines and other experimental tissues such as xenograft tumours or tissues from animal models. TMAs are also an ideal approach to organize large tissue repositories, such as entire archives from pathology institutes, enabling tissues having a specific molecular pattern to be found easily. A TMA containing samples from the formalin-fixed, paraffin-embedded specimens of these tumours, of which frozen material is also available, would help to identify rapidly tumours that are suited for very specific research projects. For example, the availability of an array of breast carcinomas of which unfixed material was available enabled us to identify rapidly tumours having 17q23 amplification, which could then be used to examine the expression of multiple different candidate amplification target genes [11].

TMAs in quality control, standardization, education, and tissue anonymization

The literature on the expression of proteins in tumours is often quite conflicting. Differences in the antibodies used, staining protocols, fixation of tissues, selection of patients, and criteria for the interpretation of staining are routinely discussed in these papers as possible sources for a surprising variability of results. Once such studies are executed on TMAs, it will be easy to determine the nature of inter-laboratory discrepancies. Exchanging unstained slides between laboratories that have reported conflicting data would rapidly allow us to see whether experimental differences caused the problem. Exchanging stained sections (either in real or as images) would easily clarify whether different modes of interpretation contributed to the confusion. Even differences in tissue processing could be unmasked as a

reason for discrepancies if matched series of tumours from different laboratories were placed on one joint TMA; for example, including 100 grade 3, N+ breast tumours with a diameter of between 20 and 30 mm from each laboratory. If all participating groups had tissue arraying capability, it would be easy to generate such a joint TMA by just shipping a recipient block from laboratory to laboratory, while each group added their specified set of tumours to the TMA block.

TMAs are also ideally suited for educational purposes. While the interpretation of large sections always reflects an attempt to integrate the observations in multiple different regions of a tissue section, the reading of TMAs is much easier and therefore more reproducible. In TMAs, morphological classifications and interpretations of immunostaining are based on the findings within one small, highly defined tissue area. Therefore, the criteria for diagnostic decision-making can easily be applied and learned on TMAs. Examples for analyses that could be trained on TMAs include the histological grading of urinary bladder tumours and the interpretation of HER-2 immunostaining in breast cancers. In both cases, a trainee could read a TMA slide and then be allowed to verify his then diagnostic ability by comparing his/her results with those of a recognized expert in the field and generating a survival curve based on his then own data and comparing it with the survival curve based on the expert's opinion.

In addition to these major applications, TMA technology can facilitate many small tasks in the daily life of routine pathology laboratories. TMAs can expedite all tasks requiring multiple analyses of a specified group of tissues. For example, we have recently generated a TMA containing ten MDM2 amplified tumours with and ten matched tumours without MDM2 amplification in order to test different MDM2 antibodies. TMAs are ideally suited for testing antibodies and optimizing staining protocols, especially if TMAs containing many different normal tissues are utilized.

TMAs are also an ideal tool for tissue anonymization. This is of paramount importance at times when the regulations to protect human subjects involved in research become more and more tight. TMA technology makes it possible to anonymize completely large sets of tissue samples with abundant clinical information, even for internal use. The only requirement to generate such absolutely anonymous tissue collections is to destroy the original biopsy/patient identifiers from the data file attached to a TMA once a TMA block is constructed. As soon as the survival information and pathology information are linked to a TMA coordinate, there is no need to retain information on which patient sample has been put on which array position. Destruction of this information will make it impossible to trace biological information back to a patient.

Future developments

Obviously TMAs will soon become much more available than now. This will allow scientists to include TMAs from different sources in one single study. This may not only be advisable, to maximize the number of tumours. The combination of TMAs from multiple different sources is also important to exclude bias. Despite promising results from our laboratories, it can never be excluded that TMAs from one particular source may carry a bias; for example, induced by a unique patient selection or tissue processing procedure in a given hospital, or consequent punching from the centre or the periphery of tumours. Data suggesting clinical significance for a new molecular marker will be much stronger if the same results are obtained on multiple different TMAs than if they are observed on just one TMA.

It is likely that devices allowing automated TMA construction will become available. While these machines will not significantly accelerate the generation of new TMAs, automated array construction is likely to increase the quality of array making and the throughput of operations aiming at generating a large number of identical TMAs. Therefore, the automation of arraying will further contribute to a better availability of TMAs. If TMAs are abundantly available, scientists will produce gigantic amounts of data. If one technician stains 40 sections per day and each section contains only 500 tumours, more than 400 000 immunohistochemical analyses can be performed within 1 month. Despite miniaturization, the reading of these sections will still be a considerable task for pathologists, emphasizing the need for automated analysis. The TMA technology greatly facilitates automated analyses. By default, the TMA technique solves the major obstacle for automated slide interpretation – the selection of a representative area for analysis. This has already been achieved during the selection of the regions to be arrayed. Attempts to create automated solutions for the reading of TMA staining are currently underway.

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