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The tissue micro-array as a translational research tool for biomarker profiling and validation

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Abstract
High-throughput molecular technologies have provided a wealth of putative biomarkers representing potential diagnostic, prognostic and therapeutic targets. Target validation commonly requires abundant and well-characterized tissue resources, as well as linked clinicopathologic data-sets. The tissue micro-array (TMA) is a validation and discovery platform of increasing popularity and necessity. TMAs provide a rapid means of examining *in situ* gene expression in a wide spectrum and large number of tissue samples. This paper describes the uses of TMAs as a translational research tool in biomarker profiling. The laboratory has performed numerous biomarker studies on several types of epithelial cancers. As an example, Epithelial Protein Cell Adhesion Molecule (EpCAM), a panepithelial antigen used to target tumours with immunotherapy, was examined in patients with renal cell carcinoma (RCC). EpCAM was an independent prognosticator for improved disease-specific survival in 318 clear cell RCCs examined, with a Cox proportional hazards multivariate hazard ratio of 0.63 ($p = 0.017$; 95% confidence interval 0.43–0.92). Interestingly, despite a typically wide-spread epithelial expression, EpCAM is instead infrequently expressed in clear cell RCC, the most common type of renal cancer, making it a poor target for immunotherapy. In this capacity the TMA provided strong support for halting lengthy and costly clinical trials for this application.

Keywords: *Tissue micro-array, EpCAM, renal cell, carcinoma, tumor biomarkers*

Introduction

A number of high throughput array techniques have evolved for monitoring and comparing gene expression in cells and tissues in diseased vs normal states. Most of these technologies depend on attaching cDNA or oligonucleotides to array chips or glass slides. While powerful, this approach lacks the ability to examine genetic events in a diverse set of cells or tissues in the context of *in situ* morphology. DNA arrays are useful discovery-based platforms that yield abundant data suggesting the involvement of putative biomarkers in disease, but they are insufficient validation platforms. Biomarker validation requires close investigation of each biomarker across a wide spectrum of disease states in, typically, hundreds to thousands of patients.

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Tissue micro-array background

More recently, in response to this need, new high throughput techniques have been developed to rapidly analyse numerous tissue samples. One such technology, the 'Tissue Micro-array' (TMA), allows rapid *in situ* screening of hundreds or thousands of samples simultaneously and the ability to perform comparative analyses from serial sections of the same array. The high-density TMA technique was developed in the laboratory of Olli-Pekka Kallioniemi at the National Institutes of Health in 1998 (Kononen et al. 1998). The machines developed to produce these precision TMAs are now commercially available through Beecher Instruments (Sun Prairie, WI).

The principle of this machine is that 'cores' of tissue (0.6–2.0 mm in diameter) are removed from paraffin embedded 'donor' blocks. Multiple cores are then arrayed into a new 'recipient' block of paraffin, which ultimately becomes the TMA. In a single standard TMA block, up to 1000 cylindrical tissues samples can be arrayed, though, more commonly, ~500 are placed per block. From one completed TMA block hundreds of array slides can be produced, each with hundreds of tissue samples ready for assay (Figure 1). While the tissue sample number is high, importantly, the amount of tissue taken from the donor blocks to make the TMA is quite small. Therefore, archival specimens are better conserved compared to conventional analyses utilizing whole sections from the same donor blocks. Assay time and reagents are drastically reduced as well since the number of TMA slides required for each assay is typically very small. While 0.6 mm cores are in widest use, 1.0, 1.5 and 2.0 mm cores may also be used where studies call for a more abundant view of tissue morphology. However, even at 0.6 mm, there is typically sufficient cellular materials and architecture present for analysis (Figure 2).

The TMA slides can be used for *in situ* detection of inter-phase DNA segments by fluorescence *in situ* hybridization (FISH) and for protein-level immunohistochemical (IHC) studies (Figure 3), as well as for *in situ* PCR and RT-PCR. These studies may be done separately or, more powerfully, in parallel (for example, to concurrently compare gene amplification and expression in the same tissues). In addition, the

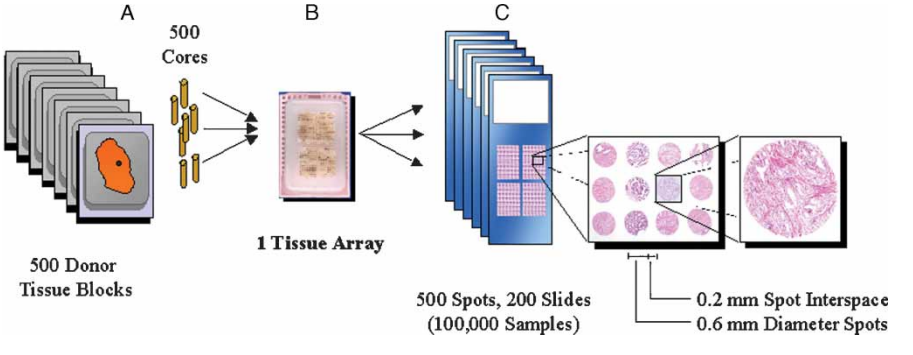

Figure 1. Method of tissue array production (*a*) Paraffin-embedded tissue cores are obtained from a set of 'donor' tissue blocks and inserted into a 'recipient' tissue micro-array (TMA) block (*b*), using a thin-walled hollow needle and stylet. The TMA block is sectioned and the resultant paraffin section is transferred to the glass histologic slide (*c*), now ready for assay. The haematoxylin and eosin stained tissue spots shown at increasing magnification on the right depict the overall placement of the TMA tissue elements, here 0.6 mm diameter cores are used, arrayed with a 0.2 mm inter-space.

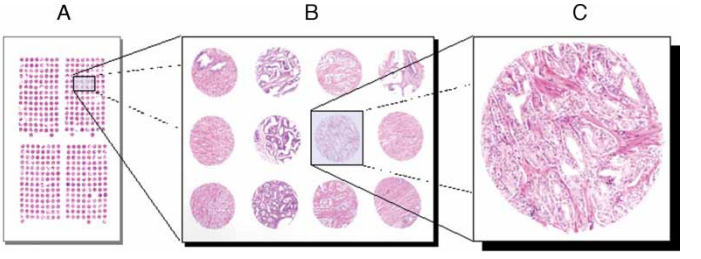

Figure 2. Close-up of tissue micro-array. Haematoxylin and eosin stained example of a prostate tissue micro-array produced by the UCLA Tissue Array Core facility, shown at increasing brightfield magnification. A single 0.6 mm TMA 'spot' shows intact tumour architecture and abundant tumour cells for study (captured with (*a*) a flatbed scanner and (*b*) and (*c*) a brightfield microscope with 2 × and 10 × objectives, respectively).

arrays provide a lasting resource tool for future studies. Therefore, the large number of available replicate TMA slides allows consecutive analysis of numerous molecular markers from related or unrelated studies. Rich gene expression datasets are, thus, produced and are amenable to data mining techniques.

The platform has been successfully validated in a number of studies comparing results from TMAs vs conventional whole tissue sections (Camp et al. 2000, Gillett et al. 2000, Hoos et al. 2001, Nocito et al. 2001, Rubin et al. 2002) The UCLA Tissue Array Core Facility constructs what are referred to as intelligent, high-density tissue micro-arrays. 'High density' refers to the array consisting of small, tightly spaced samples from many hundreds of patients. 'Intelligent' refers to the associated databases consisting of extensive pathology and clinical data linked to each array spot. It is the critical triad of pathology, clinical and gene expression data that makes the TMA such a powerful biomarker validation tool.

There are several uses for TMAs in biomarker validation. In particular, studies may first define the biomarker distribution as a screening assay across organ systems to define the potential therapeutic markets and also any potential for organ toxicity due to targeted therapeutics. Next, the TMA may be used to examine the relationship of a biomarker to the prognosis of disease (recurrence and/or survival), to disease prevalence (frequency and distribution in a specific patient population), to types of disease progression (e.g. increasing disease grades and/or stages; normal vs tumour; primary vs metastases; hormone sensitive vs resistant, etc.) or to therapeutic guidance (response prediction). Each study is best performed with TMAs built to analyse those

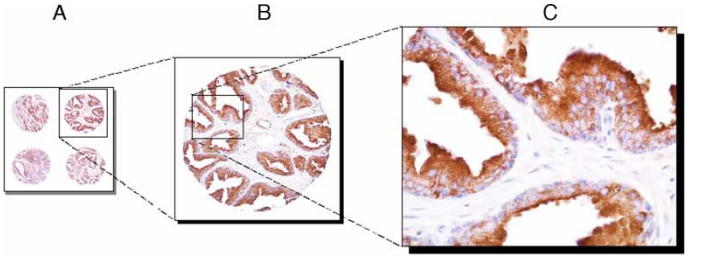

Figure 3. PTEN protein expression in prostate cancer. Immunohistochemical staining of the UCLA tissue array core facility prostate cancer TMA using an anti-human PTEN antibody (captured with a brightfield microscope with: (*a*) 2 ×, (*b*) 10 × and (*c*) 40 × objectives).

specific end-points. These studies may be expanded to include several grouped biomarkers for molecular 'profiling' where the determination of an expression signature from multiple gene products theoretically provides a closer association with these clinical parameters than any single marker could. Finally, TMAs may serve to elucidate patient groups for entry into clinical trials and serve as a platform to collect patient materials during clinical trials. For example, to collect tissues from patient groups before and after treatment for later study.

Representative biomarker study

Renal cell carcinoma (RCC) is a devastating disease. Metastatic disease accompanies one-third of new diagnoses and ~30% of those treated for localized disease eventually relapse (Figlin 1999). There are limited treatment options available and post-operative prognostication of patients treated with radical nephrectomy remains largely based on traditional clinicopathologic variables including tumour stage and nuclear grade (Fuhrman et al. 1982, Helpap 1992, Ficarra et al. 2001, Tsui et al. 2000, Bretheau et al. 1995). However, because of RCCs complex and uncertain disease course, molecular tumour biomarkers will become increasingly important adjuncts to patient care.

Within RCC, the clear cell sub-type predominates, encompassing ~70% of all RCC (Storkel et al. 1997). Epithelial cell adhesion molecule (EpCAM) is expressed in a wide variety of normal and malignant epithelial tissues including those from colon, lung, stomach, pancreas, thyroid, breast, ovary, cervix, bladder and prostate (reviewed in Balzar et al. 1999). Significant attention has also been directed to the molecule as a target for immunotherapy which has been used to reduce mortality rates for patients with minimal residual colorectal cancer approved for clinical use in Germany (Riethmuller et al. 1998). An immunocytokine fusion protein consisting of a monoclonal antibody to EpCAM linked to an active interleukin (IL)-2 molecule has been designed for the targeted delivery of IL-2 to EpCAM-expressing tumour tissues. This holds specific promise for treatment of RCC.

At UCLA, the renal TMA was used to examine the prognostic significance of EpCAM expression and its distribution in RCC tissues from 417 patients with various sub-types of RCC, to investigate the potential for anti-EpCAM-targeted immunotherapy (Seligson et al. 2004). EpCAM was consistently expressed in the distal nephron of normal renal epithelium, with 99% of samples displaying positive staining in greater than or equal to 50% of their cells. While 45% of papillary RCC, 100% of chromophobe RCC and 71% of collecting duct RCC stained to this level, only 13% of clear cell RCC samples expressed as robustly and 59% of clear cell RCC cases were considered negative altogether (Figure 4).

Because of the consistent high expression in morphologically normal renal tissues and minimal expression in the most common RCC sub-type, clear cell carcinoma, targeted treatment using EpCAM is likely of limited utility and costly clinical trials for this purpose are not recommended. However, while EpCAM was not found to be a useful therapeutic target, it was found to be an independent prognostic factor for improved disease-specific survival against conventional prognosticators, with a multivariate Cox proportional hazards hazard ratio of 0.63 ($p = 0.017$; 95% confidence interval 0.43–0.92). Therefore, the presence of EpCAM was a protective finding for patients with clear cell carcinoma. The TMA rapidly surveyed the

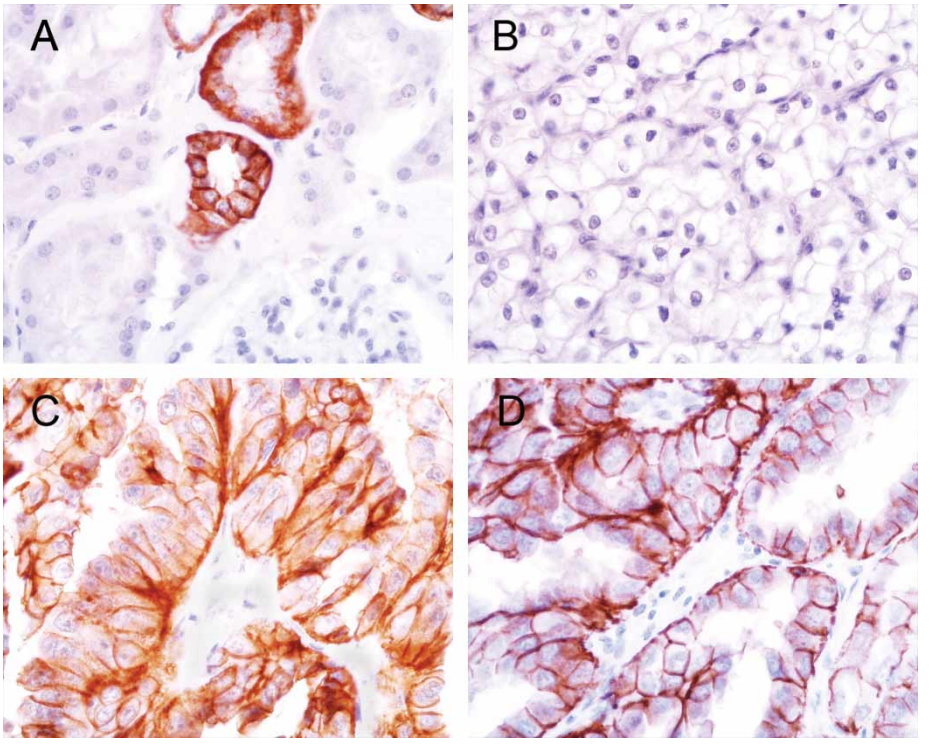

Figure 4. EpCAM protein expression in normal and malignant renal tissues by immunohistochemistry on tissue micro-arrays. (*a*) Matched morphological normal kidney with strong staining corresponding to distal convoluted tubules and relative negativity of other parenchymal components. (*b*) Clear cell RCC with negative staining. (*c*) Papillary eosinophilic RCC with strong basolateral staining. (*d*) Collecting duct RCC with strong basolateral staining (original objective: (*a*–*d*) 40 ×).

spectrum of human RCC and provided validation that EpCAM was not a promising therapeutic target in common RCC, but was indeed a prognostic factor.

Conclusion

The TMA is a powerful platform for biomarker analysis and validation that is finding increasing use in academic and industrial centres. It maximizes the utility of limited and precious tissue resources and speeds assay through-put. In the future, automated image analysis with morphologic feature identification will further accelerate analyses on this useful platform.

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