

## Review Article

# Use of laser capture microdissection, cDNA microarrays, and tissue microarrays in advancing our understanding of prostate cancer

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## Abstract

**One difficulty in studying epithelial tumors has been the inability to isolate pure samples for DNA and RNA analysis. Prostate cancer, with its infiltrative nature, is particularly challenging. The combination of several new technologies should help overcome these hurdles. Laser capture microdissection uses a laser beam to achieve transfer of pure cell populations for isolation of DNA, RNA, and protein. High-throughput analysis of these samples can be achieved by using cDNA expression microarrays. High-density tissue microarrays should allow for validation of differentially expressed genes. This review will concentrate on the application of laser capture microdissection, cDNA microarrays, and tissue microarrays in the area of prostate cancer research. Copyright © 2001 John Wiley & Sons, Ltd.**

**Keywords:** prostate cancer; microarray; laser capture microdissection; microdissection

## Introduction

One difficulty in studying epithelial tumours has been the inability to isolate pure samples for DNA and RNA analysis. Prostate cancer (PCA), with its infiltrative nature, is particularly difficult. In the past, most molecular studies have concentrated on the analysis of bulk tissue samples. That is, samples that were collected without careful pre-dissection of other tissue elements, including supporting stroma, microvasculature, and inflammatory cells. Several new technologies, however, allow us to overcome these hurdles and add in the precise evaluation of near-pure samples of benign or malignant cell populations.

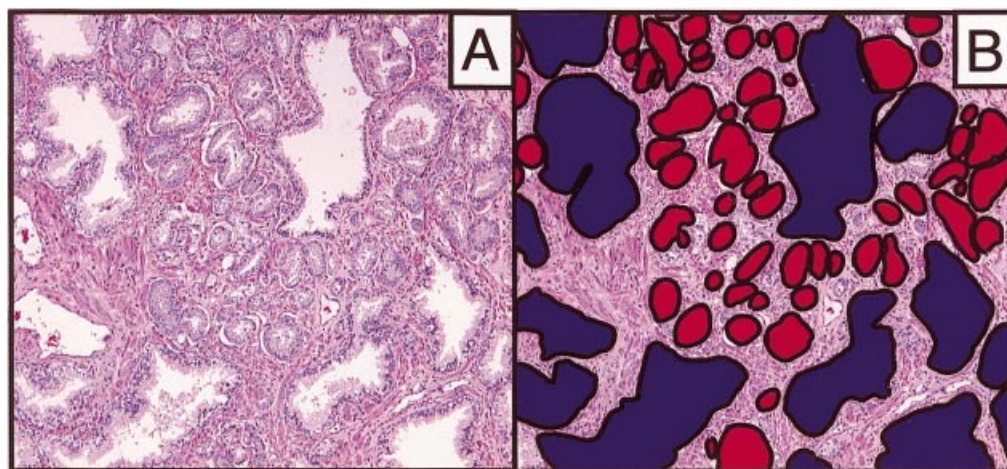
These technologies include laser capture microdissection (LCM), which was developed at the National Cancer Institute at the National Institutes of Health (Bethesda, MD, USA) and initially described by Emmert-Buck *et al.* [1]. LCM allows for the precise isolation of individual cells of interest using a microscope to guide laser transfer of the desired tissues. Once the tissues have been successfully transferred, molecular analysis, including DNA and RNA extraction, is possible. The second major high-throughput technology which should offer tremendous insight into the field of cancer biology and, in particular, PCA research is the development of cDNA microarrays. These arrays allow for the simultaneous evaluation of thousands of genes from different populations. By using cDNA microarray technology, one can assess the over- and under-expression of genes in a tumour population compared with normal populations of cells. The third technology, which can be used in close association with cDNA microarrays, is high-density tissue microarrays.

High-density tissue microarrays, as initially described by Kononen *et al.* [2], allow for the evaluation of hundreds of formalin-fixed, paraffin-embedded tissue samples (e.g. benign, dysplasia, tumour) on a single standard glass slide. This high-throughput method to evaluate protein expression is extremely important, given the large number of candidate genes discovered through cDNA microarray technology.

This review will concentrate, then, on the application of LCM, cDNA microarrays, and tissue microarrays in the area of PCA research.

## Laser capture microdissection and its role in the evaluation of prostate cancer

One of the major goals of the Cancer Genome Anatomy Project (C-GAP) is the construction of cDNA libraries from pathological tissue [3]. It was recognized early that two main sources of tissue were available for creating cDNA libraries. The first source was bulk tissue cDNA from RNA derived from large tumours. This strategy was appropriate for certain tumour types, such as lung, colon, and ovary, that are most often surgically resected as mass lesions that are grossly identifiable. However, it was recognized that regardless of tumour type, bulk tissue, even in its highest purity, still has potential contamination from stromal, endothelial, and inflammatory cells. This is a particular problem in the evaluation of PCA (prostate cancer). The prostate gland is a complex organ that is composed of epithelial and stromal cells. The normal epithelial component often represents only a small percentage (5–10%) of the entire prostate. PCAs are often seen to grow in an infiltrative pattern, with individual tumour acini infiltrating through stroma and directly adjacent to benign prostatic

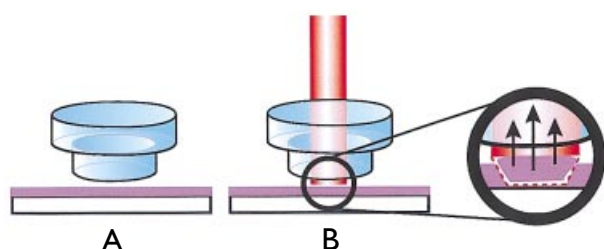


**Figure 1.** The infiltrative nature of prostate cancer makes the isolation of pure tumour samples difficult. (A) Example of a localized prostate cancer; (B) tumour cells (red) are seen in close association with benign glands (blue) (A, B)  $\times 100$

epithelium (Figure 1). Therefore, a second approach for collecting pathological tissues in the C-GAP project was to use the newly developed LCM technique to develop a cDNA library [4,5].

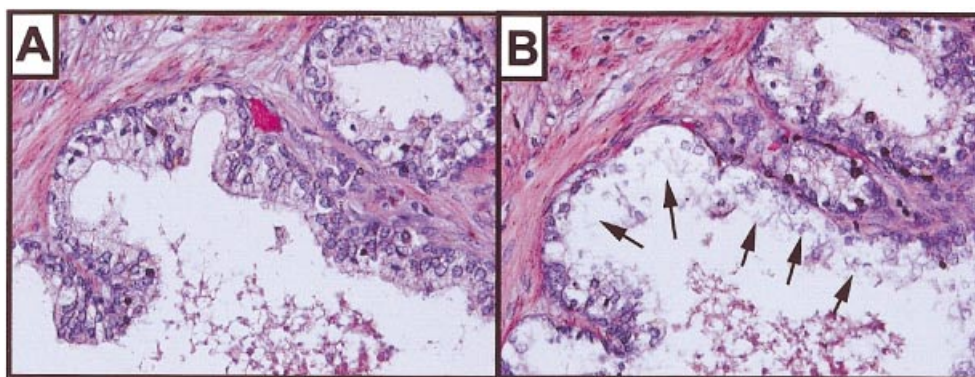
LCM was developed by Emmert-Buck *et al.* at the National Cancer Institute (NCI) of the National Institutes of Health (Bethesda, MD, USA) [1]. LCM was born out of a need to isolate pure populations of tumour, normal, and dysplastic tissues for the C-GAP

project. The most widely used LCM device is the apparatus developed by the NCI and commercially distributed by Arcturus Engineering (Mountain View, CA, USA). In this version, LCM uses a laser beam and a special thermoplastic polymer transfer film, which is bonded to the underside of a transfer cap (see Figure 2A). The cap is set on the surface of the tissue and a laser pulse is sent through the transparent cap, expanding the thermoplastic polymer. The selected cells adhere to the transfer cap and can be lifted off the tissue and placed directly into an Eppendorf tube for extraction (Figure 2B). Precise LCM can achieve transfer of pure cell populations (Figure 3).



**Figure 2.** Laser capture microdissection (LCM) uses a laser beam and a special thermoplastic polymer transfer film, which is bonded to the underside of a transfer cap (see A). The cap is set on the surface of the tissue and a laser pulse is sent through the transparent cap, expanding the thermoplastic polymer. The selected cells are now adherent to the transfer cap and can be lifted off the tissue and placed directly onto an Eppendorf tube for extraction (B)

LCM represents a major advance over previous methods of microdissection, which often used a 30-gauge or smaller needle to dislodge tumour under direct bright field visualization [6]. LCM has now been used successfully for numerous studies in the field of prostate [7–10], breast [11], lymphoma [12–14], and lung [15,16] cancer research. In the field of PCA research, the initial studies have used LCM to evaluate molecular alterations at the DNA level. Lutchman *et al.* were interested in analysing dermatin, a cytoskeleton protein, which is encoded by a gene on chromosome 8p21.1 [17]. They employed LCM to



**Figure 3.** LCM can isolate pure populations of cells. In this example, benign secretory cells are successfully transferred, leaving behind basal cells (arrows). (A, B)  $\times 200$

identify abnormalities at the locus of interest using loss of heterozygosity analysis from laser capture microdissected samples of prostate tissues. A second study by Rubin *et al.* required the use of LCM to study loss of heterozygosity at 10q23.3, a region that has been associated with multiple tumours, including glioblastoma multiforma, melanoma, endometrial carcinoma, and PCA [10]. 10q23 was also the site of *PTEN/MMAC1*, which has been found to be mutated in prostate cancer cell lines, xenographs, and hormone refractory PCA tissue specimens. This study required using several microsatellite markers in the region of 10q23. The results indicated an increase in genetic alterations as determined by loss of heterozygosity analysis in lymph node-positive prostate cancers (pT2–3, N+), suggesting that 10q23.3 is an important region in the development of metastatic PCA. What was critical to the successful completion of this study was the ability to isolate pure tumour samples from cases of clinically localized PCA, where often the tumour was either small in size or extremely infiltrative in nature. These types of loss of heterozygosity analysis studies are similar to those that have been done in the past using microdissection with 30-gauge needles [6]. However, there are two important differences. The first is that the PCA isolated using LCM is much purer; of the order of 95–100% pure PCA samples can be isolated. The second difference is that without LCM, a significant percentage of PCA samples could not be used. In the Rubin *et al.* study, probably 30–40% of the cases could only be microdissected using LCM [10]. This means that without LCM, a large percentage of smaller tumours would need to be excluded from the molecular analysis, tending to bias the study of larger or bulky tumours.

More recently, LCM has been successfully used to extract mRNA from frozen tissues [1,5,18–22]. Fend *et al.* [23] developed a rapid immunostaining method of frozen sections to allow for an ultra-specific LCM of frozen tissues. This technical advance might be useful, as specific cell subtypes are needed, such as basal cells in prostate or various subcomponents of an inflammatory infiltrate (e.g. T- or B-cells). This protocol may also be useful in the identification and isolation of cells from a similar population, which differ by their metabolic state. For example, one could do a study on PCA cells that are proliferative as determined by Ki-67 (MIB-1) immunostaining. This method may also be useful in identifying cells that are morphologically difficult to identify using standard LCM protocols. The standard protocol requires using an un-overslipped frozen section (Arcturus Engineering, Mountain View, CA, USA). Therefore, the use of a rapid immunohistochemistry method, which might take an additional 12–25 minutes, allows for a strong contrast provided by the DAB (chromogen) staining.

LCM has also been used in a proteomic approach to identify intracellular protein within PCA and normal secretory cells [8]. Ornstein *et al.* used one-dimensional and two-dimensional polyacrylamide gel electrophoresis (PAGE) on cellular lysates from LCM isolated

prostate cells. They found that prostate-specific antigen (PSA) existed in the free (unbound) form in both PCA and normal prostate secretory cells. They concluded that binding of PSA to alpha-1-antichymotrypsin occurs outside the cell and is not different between prostate cancer and benign cells. Ornstein *et al.*, in another proteomic study, used LCM of human prostate cancer cells to look for quantitative and qualitative alterations in protein [7]. This particular approach is required because of previously mentioned contamination from inflammatory cells, vessels, and non-malignant epithelial cells. They were able to use 8 µm thick frozen section slides to extract protein, estimating a transfer (e.g. dissection) of 40–50 000 cells at a level of 95% or greater purity. The cells were then lysed and two-dimensional PAGE was performed overnight. The results were digitally scored, which allowed for a comparison of protein fingerprints between PCA and normal cells. Only spots that were unique to either PCA or normal prostate were considered altered. In their initial studies, less than 98% of the proteins were determined to be identical in both benign and malignant populations. However, six distinct tumour-specific proteins were identified. The approach is successful in identifying unique sets of proteins that could potentially be used for clinically relevant serum tests. Another important finding in this study was the discrepancy between *in vivo* PCA and prostate tumour cell line (i.e. LNCaP and PC-3) protein expression. They highlight the limited usefulness on data gathered on serially passaged tumour cell lines.

Other novel approaches using single-cell LCM and proteomics have identified ways of performing analytical measurements of protein number in laser capture microdissected cells [9].

LCM has been used successfully to extract DNA, mRNA, and to perform proteomics. All of these studies are technically difficult and require the most stringent of conditions in order to ensure reproducible results. However, the potential benefit of determining genetic alterations that are specific to a selected and pure population of cells is significantly more informative than molecular analysis performed on bulk tissue samples.

### **cDNA expression arrays and high-density tissue microarrays**

One of the new emerging technologies in the post-genomic era are cDNA expression microarrays. These arrays can carry over 50 000 genes, which can be analysed in a single experiment. This approach allows for the evaluation of all potential candidate genes and pathways that may be related to tumour progression or metastasis. This approach should allow for rapid results, which otherwise would have taken years and exhausted existing tissues banks [24]. Initial expression array studies in the field of PCA have been performed on tumour cell lines, primarily due to the difficulty in isolating pure tumour samples. Vaarala *et al.* examined cDNA expression between an androgen-dependent and an androgen-independent LNCaP prostate tumour cell

line [25]. In their experiment, they examined 7075 human cDNAs, which were evaluated for over- and under-expression. They found that in the hormone-sensitive LNCaP cell lines several genes, such as Kallikrein 3 (PSA), Kallikrein 2 (hK2), and androgen receptor, were overexpressed. Genes were reported as overexpressed in hormone-insensitive LNCaP cells and these included TNF receptor-associated factor-binding protein, TNF receptor-associated factor 3, an EST similar to galectin-1, and folistatin. The latter two gene products have previously been associated with hormone refractory PCA. These results are interesting because they confirm a discrete set of differentially expressed genes in two PCA cell lines that differ in their ability to grow in the absence of androgen. Carlisle *et al.* used a similar approach to evaluate a prostate cell line, 8.4, and a melanoma cell line, UACC903 [26]. This experiment evaluated 5184 cDNAs, which were spotted onto a nylon membrane filter. EST sequences derived from prostate cDNA libraries were used. This work was part of the C-GAP project. The key findings in this study were first to evaluate how reproducible results were from similar cell lines. They demonstrated excellent correlation coefficients when comparing results for two separate hybridizations of the same cell lines ( $R=0.9$ ). When they compared differences between the prostate and melanoma cell lines, they found that 89% of the genes showed overexpression for the prostate cell line 8.4. The melanoma cell line, which one would anticipate to have far fewer similarities with the original prostate cDNA libraries, had only 28% of the cDNAs which were expressed. Overexpression of housekeeping genes such as keratin in the PCA cell lines, but not the melanoma cell lines, confirmed the results.

Bubendorf *et al.* combined cDNA and tissue microarray technologies (see below) to examine differences in gene expression between well-characterized PCA cell lines [24]. They examined gene profile differences between hormone-sensitive CWR22 and hormone refractory CWR22R PCA cell lines. The results were validated on a tissue microarray of specimens taken from a broad spectrum of patients with PCA. Two types of commercially available cDNA arrays were used for the purpose of this experiment (Clontech Laboratories, Inc., Palo Alto, CA and Research Genetics, Inc., Huntsville, AL, USA) with a total of 5184 genes. A discrete set of genes (0.7% or 37 of 5184) were up-regulated in the hormone-resistant PCA cell line with more than a two-fold increase and 2.6% (135/5184) of genes were down-regulated by more than 50%. Insulin-like growth factor binding protein 2 (IGFBP2) and the 27 kd heat-shock protein (HSP27) were among the most consistently overexpressed genes in the population of hormone refractory cells (CWR22R). These two cDNA microarray results were confirmed using immunohistochemistry on high-density tissue microarrays. IGFBP2 was expressed in 100% of the hormone refractory PCAs, 36% of the clinically localized PCA, and 0% of benign prostatic tissue (two-sided  $p$  value=0.0001). For HSP27, overexpression was seen in 31% of the hormone-resistant PCA

versus 5% of the clinically localized PCA and 0% of benign prostatic tissue (two-sided  $p$  value=0.0001). Overall, they found 170 genes or 3.3% differentially expressed (i.e. over- or under-expressed). This level of molecular complexity will be important to evaluate in clinical samples. They also noted that it remains critically important not only to screen for expression of many different genes, but also to screen many different tumour tissues and establish an accurate frequency of involvement of these genes in the different stages of PCA progression.

Two additional studies are noteworthy in their ability to link LCM with expression array analysis. Neither of these studies was performed in prostate; however, their approaches are important because they were able to perform cDNA analysis of LCM isolated breast and neural cells. In the first experiment, Luo *et al.* examined gene expression profiles of neural tissues from LCM isolated samples [27]. RNA amplification was performed using a linear amplification with the T7 RNA polymerase. Validation was performed using several more standard methodologies including quantitative RT-PCR and northern blot analysis to demonstrate protein expression differences. They were able to identify different gene expression profiles for histologically distinct neural populations. In another study examining breast cancer, Sgroi *et al.* compared cDNA expression from LCM breast cancers [28]. They used a custom-made nylon membrane cDNA array and examined a total of 8084 genes. Total RNA was extracted using LCM. They estimated that a total of  $1.7 \times 10^4$  to  $2.0 \times 10^4$  cells were used for RNA extraction. These cells were reverse-transcribed and labelled with  $^{33}\text{P}$ . They divided the RNA into several pools, which were used to verify overexpressed genes using RTQ-PCR. Alternatively, protein expression was also confirmed using immunohistochemistry in the cases where antibodies were available. In this study, 90 genes had significantly altered levels of expression (two-fold or greater). When they compared gene expression differences from metastatic and invasive cancers with that of normal breast, they found overexpression of such genes as apolipoprotein-D (15.2-fold increase), BRACA1 (3.8-fold increase), heat shock factor protein-1 (6.4-fold increase), and annexin-1 (21.7-fold increase). There were also numerous ESTs that were overexpressed.

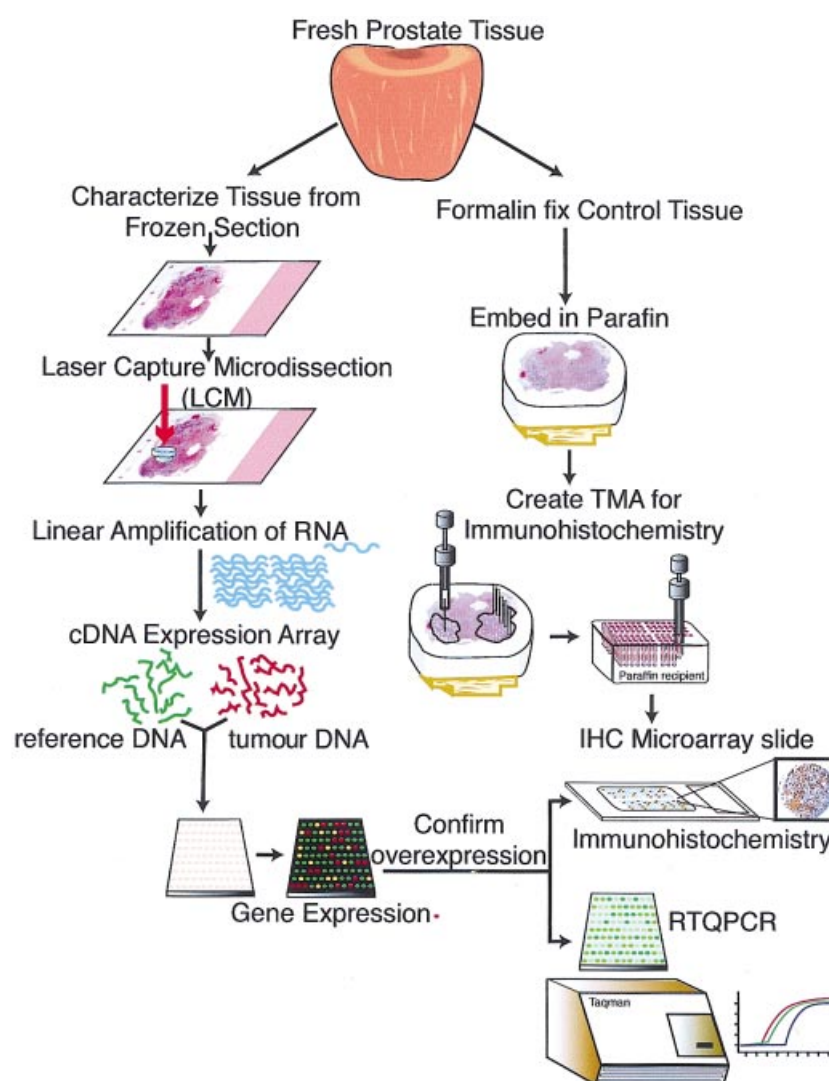
The linking of high-density tissue microarrays with cDNA microarray technology offers a way to confirm differentially expressed genes in a large number of clinical samples without exhausting banked archival tissue, which is a real concern at academic research centres. High-density tissue microarrays are the subject of another review article [29] in this issue of the journal; therefore this discussion will be limited to the use of tissue microarrays for the study of PCA. One of the first studies in PCA using tissue microarrays was performed by Bubendorf *et al.* [30]. They examined amplification of androgen receptor, cyclin D1, and cMyc using fluorescence *in situ* hybridization (FISH) on tissue microarrays. They found that both Myc and androgen receptor were

overexpressed in hormone refractory PCA with respect to clinically localized hormone-sensitive PCA. Some advantages of their approach included the successful analysis performed on one slide with 32 samples of BPH, 223 samples of primary PCA, 54 locally recurrent PCAs, and 62 metastatic PCAs from patients with hormone refractory PCA. In total, 371 samples were examined with five different probes.

Bubendorf *et al.* did not find c-erbB2/Her2neu amplification in any of the prostate samples examined [24]. These results were consistent with two previous studies which used standard slides [31,32]. However, Ross *et al.* did find high amplification of c-erbB2/Her2neu in two published studies [33,34]. The four previous studies were all performed on standard slides. Therefore one of the major advantages of the tissue microarray approach was that using a small amount of tissue in a single experiment, they were able to identify the low probability that c-erbB2/Her2neu plays an

important role in the progression of PCA. This initial study would have made it relatively unnecessary to repeat them in studies using standard slides.

The small size of the samples used for tissue microarrays has been discussed in several recent papers [2,35,36]. Some have criticized the small size of the tissue samples used for tissue microarrays. The major concern has to do with the lack of representation of the original tumour from which the sample comes. However, it is important to point out that the question being asked is not what the protein expression or gene amplification is for an individual patient to determine treatment, but instead to profile the expression of a large population of PCA tumours. The result should indicate whether a specific protein is overexpressed in the population of interest, such as hormone refractory PCA. If low expression is observed in hundreds of samples, there is a very high probability that this is a good estimate of what one would see



**Figure 4.** Schematic representation of a high-throughput approach performing precise molecular profiling of prostate cancer. The prostate gland is immediately retrieved from the operating room. After appropriate prostate tissue is taken for pathological tumour staging, frozen tissues samples are acquired for research. Laser capture microdissection is performed and total RNA is extracted and after a linear amplification, is labelled and hybridized on a cDNA microarray. The results are evaluated and important candidate genes are confirmed using quantitative RT-PCR or high-density tissue microarrays with various stages of prostate tumours

using standard slides. It is also important to point out that due to the large number of candidate genes that need to be examined, the tissue microarray approach provides an excellent way to conserve tissue. Tissue microarray technology is a powerful tool for the molecular profiling of large numbers of tumours representing the entire disease spectrum of human prostate cancer progression *in vivo* [24].

The use of LCM in combination with cDNA microarrays and confirmation using high-density tissue microarrays is an intriguing approach (Figure 4). However, limitations will be in our ability to analyse these data. The large number of genes that will be identified as being differentially expressed will require the development of databases that will allow us to categorize results. Therefore, not only will we need to know the specific genes and their function, but we will also need to be able to group the results in meaningful pathways that will be helpful in identifying tumour-specific pathways relevant in the progression of PCA. Due to the difficulties in analysing gene expression results, Kaldjian and Giesig (unpublished study) have employed a system of 'functional taxonomy' to categorize the data. Selected genes are placed into 16 different cellular function categories that are further divided into subcategories. For each sample, the number of genes in each category can be calculated and graphed, providing a cellular function fingerprint of gene expression. This 'functional taxonomy' approach will allow us to deal with cDNA expression array results from numerous experiments from many patients. By taking this approach, one would hope that after the analysis of 50–100 tumours, patterns would develop. As clinical data are linked to these profiles, outcomes such as PSA biochemical failure, development of metastatic disease, or death may demonstrate unique profiles. Response to treatment may also be linked to a specific functional profile.

From a practical standpoint, none of the studies using these new technologies has identified any clinically significant genes or groups of genes that have made their way to the bedside. However, we are at the very early phases of this technology; advances are anticipated in both the high-throughput analysis and database development, which should dramatically increase our likelihood of finding clinically significant genes or groups of genes that will translate into diagnostic or therapeutic modalities for men with PCA.

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