Use of High-Throughput DNA Microarrays to Identify Biomarkers for Bladder Cancer

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Background: Numerous markers have been described to correlate to some extent with tumor stage and prognosis of patients with bladder cancer. The power of many of these biomarkers in detecting superficial disease or predicting the clinical outcome of individual tumors is limited, and alternative markers are still in demand. High-throughput microarrays represent novel means for cancer research and tumor marker discovery.

Approach: The aim of this report was to discuss the application of DNA technologies to provide novel biomarkers for bladder cancer.

Content: Specific bladder tumor subtypes have distinct gene expression profiles. The use of high-throughput DNA microarrays allows identification of the most prevalent and relevant alterations within bladder tumors. Clusters of differentially expressed genes will become biomarkers to discriminate subgroups of patients with different histopathology or clinical outcome. Additionally, the identified individual molecular targets might be further validated and developed into novel serum or urinary biomarkers for the diagnosis and/or as prognostic factors to be applied in clinical practice. The diagnosis and prognosis of bladder cancer would be enhanced by the use of such markers, and the marker itself may constitute a therapeutic target when studied in appropriate patients and control groups.

Summary: Expression profiling with high-throughput DNA microarrays has the potential of providing critical clues for the management of bladder cancer patients. As the quality, standardization, and ease of use of the technology increase and the costs decrease, DNA microarrays will move from being a technology restricted to research to clinical laboratories in the near future.

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Bladder Cancer: Pathologic and Clinical Features

Bladder cancer is the sixth most common malignancies in developed countries, where most bladder tumors are transitional cell carcinomas (TCCs)(1). In contrast, squamous cell carcinoma of the bladder is the predominant presentation in Middle Eastern countries (2). Approximately 75% of uroepithelial TCCs are superficial (TIS, Ta, and T1), 20% are muscle infiltrating (T2–T4), and 5% are metastatic at the time of diagnosis (1). Most superficial cases are treated conservatively by transurethral removal of the tumor, followed by adjuvant intravesical therapy, but without removal of the bladder. Muscle-infiltrating tumors are generally treated by cystectomy plus systemic adjuvant chemotherapy or radiotherapy. Bladder cancers follow widely varying clinical courses. Of the superficial tumors, ~20% are cured by surgical removal of the presenting lesion (5 years with no evidence of disease), 50–70% recur one or more times, but do not progress into invasive disease, and 10–30% progress to invasive and potentially lethal disease (3). In contrast, ~50% of patients with muscle-infiltrating tumors harbor or will develop metastatic disease. Although recent advances in systemic chemotherapy have improved the prognosis for patients with metastatic disease, the vast majority ultimately die from their disease. Thus, it is important to identify patients whose tumors are likely to recur or progress so that they can be treated more aggressively.

The chances of superficial tumor progression are augmented with increased pathologic stage and grade, tumor size, presence of concomitant carcinoma in situ, and multicentricity (2, 3). The power of these histopathologic variables in defining the clinical subtypes of bladder cancer and predicting the clinical outcome of individual patients has certain limitations. Many groups of investigators have examined additional molecular characteristics of bladder cancer that may be of predictive value.

Bladder tumors, including some superficial lesions,
carry a substantial number of genetic alterations at the time of diagnosis (4). A large body of work has suggested that superficial papillary tumors (pTa) differ from flat carcinoma in situ lesions (TIS) and muscle-invasive tumors in their molecular pathogenesis and pathways of progression (1–8). The number of genetic alterations is substantially higher in the invasive lesions, but there also appears to be a difference in the specific alterations present. On the basis of data from several groups, it appears that at least two major molecular pathways of bladder tumor development and evolution can be followed (Fig. 1). Briefly, the first pathway, represented by papillary superficial tumors, is associated with chromosome 9 losses, including inactivation of CDKN-2 (p16) on 9p and still unknown genes associated with telomeric 9q loci (5). The second pathway includes inactivation of p53 on chromosome 17 (17p11.3) and Rb on 13q14, ascribed to flat carcinoma in situ and pT1 tumors (6, 7). Phenotypic features associated with biological tumor aggressiveness include cell cycle and apoptosis regulators studied by appropriate sensitive molecular techniques. Numerous markers have been identified that correlate to some extent with tumor stage and prognosis. However, the power of many of these markers in predicting the clinical outcome of individual tumors is limited, and alternative markers are still needed for detection of the disease and for predictive purposes.

**Urinary Tumor Biomarkers in Bladder Cancer**

The diagnosis of bladder cancer is basically based on the combined information provided by urinary cytology and cystoscopy. Unfortunately, cystoscopy does not provide 100% sensitivity for the diagnosis of bladder cancer, and it is an invasive, uncomfortable diagnostic method for many patients (2, 3). Urinary cytology as the standard noninvasive method also has sensitivity limitations, extreme dependence on sample quality, and high interobserver variability (2, 3, 8). Thus, alternative noninvasive objective methods have been developed for the diagnosis and monitoring of the disease. Urinary tumor markers represent an option of interest for the early detection and surveillance of the disease because of sample accessibility and the direct contact of urine with the tumor. Many urinary tumor markers have been evaluated in the last

![Fig. 1. Overview of genetic alterations of the two major progression pathways described for bladder cancer.](image-url)
few years (9–24); Table 1 summarizes some of the characteristics of these markers. The sensitivity overall and by stage and grade is better for most available markers compared with cytology, although they are less specific in high-grade tumors (23). Overall, urinary tumor markers could potentially replace urinary cytology in clinical daily practice (9–23). However, their overall sensitivity has not been demonstrated to be sufficient to replace cystoscopy as the gold standard. Most of the studies reported to date are descriptive evaluations of the sensitivity and specificity of these markers at the time where diagnosis is established, whereas the utility of urinary markers in monitoring of the disease has been less frequently reported (22). Multicenter studies are necessary to reach a consensus in the optimization of sample acquisition and preanalytical issues critical for reliable laboratory results, which markers are the best option for every clinical need, and when and how they should be determined (23, 24). It is our hope that the increase in bladder cancer detected in superficial preinvasive stages is attributable not only to simplified procedures, such as the use of flexible cystoscopy in combination with urinary cytology, but also to the introduction of noninvasive urinary tumor markers in the clinical setting. The use of exclusion criteria can improve the specificity of urinary tumor markers at first diagnosis and in the surveillance of bladder cancer patients (9–24). However, the lack of specificity of urinary tumor markers in certain circumstances is leading investigators to search for novel biomarkers that may display higher specificity. Candidate biomarkers include other cytokeratins (25); other nuclear matrix proteins, such as BLCA4 (26); other molecules, such as survivin (27), cyclooxygenase-2 (28), and Mcm5 (29); or the use of highly sensitive and specific techniques such as microsatellite analysis (17) or dual parametric flow cytometry (30). One potential approach involves molecular profiling of bladder tumors by high-throughput technologies (31). Developments in areas of research such as gene expression microarrays or proteomics represent novel means for cancer screening and potential urinary tumor marker discovery.

**Potential Role of High-Throughput DNA Microarrays in Identifying Biomarkers in Cancer**

Understanding the biology underlying tumorigenesis and tumor progression of bladder cancer is essential for improving the capacity to diagnose and treat the disease. Unraveling the biological complexity underlying these processes is expected to provide novel tools of predictive nature and to enable identification of therapeutic targets by selecting those molecular targets significantly differentially expressed in bladder tumors. The further characterization of regulatory mechanisms and pathways controlling cellular homeostasis that are altered in bladder tumors will be achieved, at least in part, by global analyses of gene expression. Until recently, the ability to identify and analyze gene expression patterns has been technically limited to relatively few genes per study. This limitation is being overcome by the development of several methods that allow more comprehensive analysis of these patterns. Some of the most powerful methods include differential display (32), serial analysis of gene expression (SAGE) (33), protein composition-based approaches such as protein microarrays (34), and combined two-dimensional gel electrophoresis followed by mass spectrometric analysis (35).

Concurrent with the development of these methods has been the tremendous increase in the DNA sequence information available through genome sequencing efforts (36, 37). This expansion of gene and genome information has served as the basis for development of DNA microarrays, one of the most powerful new methodologies available to provide both static and dynamic views of gene expression patterns in cells and tissues (38).

The utility of DNA in microarray format arises from the ability of single-stranded nucleic acids to hybridize with high specificity to a second strand containing the

### Table 1. Main characteristics of several of the most extensively studied urinary tumor markers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Antigen type</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Method</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMP22</td>
<td>Soluble</td>
<td>47–87</td>
<td>58–91</td>
<td>EIA</td>
<td>Matritech</td>
</tr>
<tr>
<td>BTA STAT</td>
<td>Soluble</td>
<td>57–82</td>
<td>61–82</td>
<td>Antigen-antibody colorimetric</td>
<td>Bard Diagnostics</td>
</tr>
<tr>
<td>BTA TRAK</td>
<td>Soluble</td>
<td>55–80</td>
<td>38–98</td>
<td>EIA</td>
<td>Bard Diagnostics</td>
</tr>
<tr>
<td>FDP</td>
<td>Soluble</td>
<td>41–93</td>
<td>77–94</td>
<td>Antigen-antibody colorimetric</td>
<td>Intrace Corp.</td>
</tr>
<tr>
<td>Telomerase</td>
<td>Cellular</td>
<td>53–91</td>
<td>46–99</td>
<td>PCR</td>
<td>Oncor</td>
</tr>
<tr>
<td>Hyaluronic acid/hyaluronidase</td>
<td>Soluble</td>
<td>82–92</td>
<td>83–96</td>
<td>EIA</td>
<td>Eichrom Technologies</td>
</tr>
<tr>
<td>Immunocyt</td>
<td>Cellular</td>
<td>86–95</td>
<td>79–90</td>
<td>IFI; cytology</td>
<td>Diagnocure</td>
</tr>
<tr>
<td>Quanticyt</td>
<td>Cellular</td>
<td>45–59</td>
<td>70–93</td>
<td>Morphometry</td>
<td>Gentian Scientific Software</td>
</tr>
<tr>
<td>UBC</td>
<td>Soluble</td>
<td>59–79</td>
<td>84–96</td>
<td>EIA</td>
<td>IDL</td>
</tr>
<tr>
<td>CYFRA 21-1</td>
<td>Soluble</td>
<td>74–99</td>
<td>57–78</td>
<td>ECLIA</td>
<td>Roche Diagnostics</td>
</tr>
<tr>
<td>BLCA4</td>
<td>Soluble</td>
<td>85–96</td>
<td>85–100</td>
<td>EIA</td>
<td>Eichrom Technologies</td>
</tr>
</tbody>
</table>

*a Sensitivity and specificity are taken from Lotan and Roehrborn (23).

*b Food and Drug Administration approved.

*c Preservative necessary.

*d EIA, enzyme immunoanalysis; IFI, immunofluorescence immunoassay; ECLIA, electrochemiluminescence assay.
complementary sequence, thus forming double-stranded nucleic acid molecules. The main advantage of DNA arrays is that they allow the study of multiple transcriptional events in a single experiment (38). Hybridization-based assays and the microarray format provide an extremely versatile technology. There is an increasingly broad range of applications to which microarrays have been applied, including genotyping of polymorphisms and mutations (39), determining the binding sites of DNA-binding proteins (40), and identifying structural alterations by use of arrayed comparative genome hybridization approaches (41). The most widespread use of this technology to date has been the analysis of gene expression.

Because genetic alterations and the resulting changes in gene expression are primary determinants controlling neoplastic transformation and progression, it is not surprising that expression profiling by use of high-throughput DNA microarrays has found wide application in cancer research and has the potential of providing critical clues for the management of bladder cancer patients. It is expected that specific tumor subtypes will have distinct gene expression profiles (31,38). The use of high-throughput DNA microarrays will allow identification of the most prevalent and relevant alterations within bladder tumors. Clusters of differentially expressed genes will become biomarkers themselves to discriminate subgroups of patients with different histopathologies or clinical outcomes. Additionally, the identified individual molecular targets might be further validated and developed into novel biomarkers for use in diagnosis and/or prognosis in clinical practice in the near future. The diagnosis and prognosis of bladder cancer would be enhanced by the use of such markers, and any marker may itself constitute a therapeutic target when studied in appropriate patients and control groups.

Expression profiling by use of DNA microarrays can render a variety of clusters or molecular targets depending on the experimental designs taken. For example, comparison of the expression profiles of bladder tumors vs respective healthy urothelium can provide information on the molecular events that take place during transformation. These type of studies may make it possible to assign potential functional roles to novel genes in both the signaling pathways controlling bladder cancer development and the phenotypic changes associated with that development. Additionally, elucidation of the molecular events involved in bladder tumorigenesis and tumor progression can lead to the discovery and application of novel biological markers. The output of high-throughput microarrays can be clusters of genes that define patients more likely to display superficial or invasive disease, to develop node or distant metastasis, or to display a shorter disease-free or survival time. Each of these clusters of genes can be considered a marker itself, and these expression patterns will represent the novel biomarkers of the disease, a concept that will have to be validated with comprehensive multicenter studies. Additionally, once an individual molecular target gene is found differentially expressed in bladder cancer, its potential role as a tumor marker can be studied at the RNA level by use of high-throughput tissue microarrays incorporating in situ hybridization or at the protein level by arrays incorporating immunohistochemistry (Fig. 2). This represents a high-throughput strategy to evaluate the potential clinical utility of novel biomarkers using clinically well-annotated tumors at the microanatomic level (42,43), which will be illustrated below with an example applied to bladder cancer.

An early detection research network has been established by the National Cancer Institute to coordinate research among biomarker development and validation laboratories, clinical repositories, and population-screening programs. Five consecutive phases have been proposed to guide the process of biomarker development. The first, preclinical exploratory phase identifies promising uses for the target. During the second, validation phase, the clinical assay is evaluated for detection of an established disease. Phase 3 includes retrospective longitudinal studies to define whether the biomarker can detect disease before it becomes clinical and to define criteria for a positive screen. Prospective screening is performed during phase 4, in which the ability of a test to detect the extent and characteristics of disease is evaluated and the false-positive rate for the test is determined. Cancer control is the fifth phase, in which the impact of screening on reducing the burden of disease on the population is quantified (44).

**DNA Microarray Studies in Bladder Cancer to Date**

Expression profiling studies using in vitro cultured cell lines as well as fresh or frozen clinical material have been used to gain insight into the molecular events associated with the disease. Below is a summary of these studies.

**Studies Using Cultured Cells**

This technology can be applied to gene function and pathway discovery using in vitro models. The tumor growth inhibition produced by genistein in TCCSUP, a susceptible bladder cancer cell line, was studied using DNA microarrays (45). Expression profiles were then analyzed at various time points, using cDNA chips containing 884 sequence-verified known human genes. The study reported many groups of genes with distinct expression profiles, most of them encoding for proteins that regulate cell growth or cell cycle. Interestingly, transient induction of epidermal growth factor receptor-1 was identified, and this molecular target was related to its proliferation and differentiation effects (45).

An example of the functional classification of genes by use of cultured cells with different phenotypes is the study relating the expression patterns of p53-mediated apoptosis in resistant bladder cancer cell lines vs sensitive cells (46). The ECV-304 bladder carcinoma cell line was selected for resistance to p53 by repeated infections with a p53 recombinant adenovirus, Ad5CMV-p53 (46). Its expression pattern in cDNA arrays containing 5730 genes
was compared that of with p53-sensitive ECV-304 cells. Several potential p53 transcription or related targets were identified as playing roles in cell cycle regulation, DNA repair, redox control, cell adhesion, apoptosis, and differentiation. Proline oxidase, a mitochondrial enzyme involved in the proline/pyrroline 5-carboxylate (P5C) redox cycle, was up-regulated in sensitive but not in resistant cells. Additional experiments with P5C, a proline-derived metabolite generated by proline oxidase, inhibited the proliferation and survival of resistant and sensitive cells, inducing apoptosis in both cell lines. These results suggested the involvement of proline oxidase and the proline/P5C pathway in p53-induced growth suppression and apoptosis, molecular events that had not been identified in p53 resistance in bladder cancer (46).

The expression profiles of nine bladder cancer cell lines, including T24, J82, 5637, HT-1376, RT4, SCaBER, TCCSUP, UMUC-3, and HT1197, have been compared against a pool containing equal amounts of RNA from each of these cell lines by use of cDNA arrays containing 8976 genes (47). Hierarchical clustering classified these tumor cells according to the histopathologic characteristics of the tumors from which they were derived. The squamous carcinoma cell line SCaBER was distinguished from the other cell lines obtained from transitional carcinomas. Moreover, cell lines from invasive lesions clustered together and were segregated from cell lines obtained from a metastatic and a papillary superficial tumor.

Additional analyses were directed to identifying potential targets that differentiated squamous features within bladder cancer based on the genes that were differentially expressed in SCaBER (47). Caveolin-1 and keratin 10 were differentially expressed in SCaBER and certain invasive tumor cell lines compared with RT4 cells, which are derived from a papillary superficial bladder tumor. The patterns of expression of keratin 10 and caveolin-1 were characterized in primary bladder tumors spotted on tissue microarrays and were shown to be significantly associated with the presence of squamous differentiation. Moreover, when a bootstrapping resampling technique was applied, the cells clustered based on their p53, Rb, and INK4A status. E-Cadherin, zyxin, and moesin were identified as genes differentially expressed in these clusters. Interestingly, the expression of these five genes was significantly associated with histopathologic stage and tumor grade in bladder tumors contained in

Fig. 2. The general procedure of an expression profiling experiment. The procedure includes RNA isolation from tumor biopsy and control samples, preparation of the hybridization probe using an oligonucleotide or cDNA platform, hybridization to the DNA microarray, data acquisition and analysis, and verification of the results by use of, for example, tissue microarrays. Tissue arrays represent a high-throughput methodology to validate the clinical utility of markers identified in molecular profiling studies using DNA microarrays. They can be used for immunohistochemistry at the protein level, in situ hybridization at the RNA level, or fluorescence in situ hybridization at the DNA level.
tissue microarrays. Moreover, moesin provided prognostic information regarding overall survival. These results revealed that molecular profiling clustered bladder cancer not only on the basis of histopathogenesis but also on biological criteria. Additionally, novel molecular targets identified were associated with histopathologic criteria and might become biomarkers of clinical utility in the management of patients with bladder cancer (47). This study represents an example of the strategy, outlined in Fig. 2, for use of DNA microarrays as a high-throughput approach to identify novel targets of clinical utility in the management of bladder cancer. In addition, these molecules may be validated by use of high-throughput microarrays containing well-annotated tumor samples (42, 43). Translation of individual patterns of gene expression obtained for in vitro-cultured cells to in vivo situations should be performed carefully because differences in gene expression using RT-PCR have been reported between cultured cancer cells and the tumors they were originated from (48). However, this study revealed that expression profiling of cultured cancer cells might reflect the biology underlying human tumor development.

**STUDIES USING CLINICAL MATERIAL**

To date, there have been few expression analysis reports using DNA microarrays on clinical material. The following studies show how oligonucleotides or cDNA microarrays can identify and correlate changes in the expression of specific genes and groups of genes with cancer and cancer-related phenotypes (31). The use of mutation or single-nucleotide polymorphism (SNP) chips will also be commented on.

The most extensive study dealing with molecular classification of bladder tumors published to date used oligonucleotide arrays carrying probes for 6500 genes to explore the expression patterns of superficial and invasive tumor cell suspensions (49). Using a sample size containing 36 normal urothelium biopsies and 29 bladder tumor biopsies, the authors prepared pools of cells made from normal urothelium and tumors of different stage–grade combinations, such as from pTa grade I and II and pT2 grade III and IV bladder tumors. Single-cell suspensions were prepared from cooled biopsies immediately after surgery, according to a procedure used previously for the preparation of bladder tumors cells for flow cytometry. These cell suspensions were inspected under the microscope to ensure the presence of enriched urothelial cells and similar numbers of tumor cells from each specimen (49). This pool approach may smooth individual differences, but it can dilute strong expression of relevant genes that may differentiate specific groups with different prognoses regarding recurrence, progression, or overall survival. Hierarchical clustering of gene expression not only correlated with the stage and/or grade of the samples studied but also identified several stage-characteristic and functionally related clusters concerned with cell proliferation, oncogenes and growth factors, cell adhesion, immunology, transcription, proteinases, and ribosomes (49). By organizing genes with similar expression patterns into clusters, the authors identified several functionally related genes that could be developed as tumor biomarkers for bladder cancer alone or in combination. Superficial papillary tumors showed increased amounts of transcription factor and ribosomal genes, as well as proteinases encoding genes up-regulation. In the invasive tumors, increased amounts of cell-cycle related transcripts were observed, which might reflect the increased concentrations of growth factor and oncogene transcripts found. A loss of cellular adhesion proteins was found in invasive tumors and may be related to tissue invasion and metastasis. In this study, the invading tumor cells seemed to challenge to the immune system, as reflected by an increase in immunology-related proteins (49).

The expression profiles of 15 bladder tumors have been analyzed against a pool of bladder cancer cell lines by use of cDNA microarrays containing 17 842 known genes and expressed sequence tags (ESTs) (50). The application of bootstraps and multidimensional scaling methods to the hierarchical clustering allowed classification of superficial bladder tumors vs lesions that invaded the muscular layer (Fig. 3). Critical known targets involved in bladder cancer progression, such as cytokerin 20 (25), p21 (51), and cyclin E (52), as well as novel molecular targets such as neuropilin-2 (53) were identified as differentially expressed between superficial tumors and tumors invading the muscular layer. These molecular targets were shown to be significantly associated with stage and grade. The application of tissue microarrays represents a high-throughput approach for validation of novel potential markers for bladder cancer by immunohistochemistry or fluorescence in situ hybridization in paraffin blocks [Refs. (43, 44, 52, 54, 55); Fig. 2]. Focus has intensified within this field to construct optimal frozen tissue microarrays that would enable further characterization of novel genes by in situ hybridization of ESTs and known genes for which specific antibodies are not available to study the potential clinical relevance of gene targets not previously evaluated.

Testing for mutations of the TP53 gene in bladder tumors is a valuable predictor for disease outcome. Today, TP53 mutations can be analyzed by use of commercial variant oligonucleotide chips in which the 1464 gene chip positions correspond to the p53 gene sequence. When this microarray was compared with the traditional manual dideoxy sequencing for sequencing of DNA extracted from 140 human bladder tumors, the concordance rate was 92% (56). Microarray-based sequencing is a novel option to assess TP53 mutations and represents a rapid, inexpensive method compared with conventional sequencing, with the additional advantage that the system is almost free from interference by mixtures of templates from nonpathologic and pathologic tissue. A target concentration as low as 1% in the diseased tissue can be detected in the presence of 99% wild-type sequences. The improved sensitivity reflects the fact that with the microchip sequencing technology, each spot (or
position) coated with a specific oligonucleotide tests for a unique sequence (56).

Recently, genome-wide screening using SNP arrays has been applied to DNA isolated from microdissected superficially invasive T1 and muscle-invasive T2–4 bladder tumors (57). These commercial oligonucleotide microarrays contain 1494 biallelic polymorphic sequences and can detect the loss or gain of at least one allele. The authors reported that of a genotype of 1204 loci, 343 were heterozygous in the bladder tumors under study. Allelic imbalance was detected in known areas of imbalance on chromosomes 6, 8, 9, 11, and 17, and a new area of imbalance was detected on the p arm of chromosome 6. Microsatellite analysis of T2–4 tumors and Ta tumors showed that allelic imbalance was more frequent in T2–4 tumors than in Ta or T1 tumors. However, when pairs of T1 and T2–4 tumors were analyzed from eight patients, 68% of imbalances detected in T1 tumors (146 imbalances) occurred in the subsequent T2–4 tumors (99 imbalances). Homozygous TP53 mutations were more often associated with high allelic imbalance than with low allelic imbalance. In this study, SNP arrays were shown to be feasible for high-throughput, genome-wide scanning for allelic imbalances in bladder cancer that was faster than comparable microsatellite-based analyses. Not only did the arrays confirm known areas of chromosomal losses, they may have identified areas with common allelic imbalances that could harbor potential tumor suppressors in bladder cancer (7). Although these chips are restricted to the polymorphic areas contained in the arrays, in the future, it should be possible to fabricate high-density SNP microarrays for other predefined chromosomal locations, which could make noninformative areas informative. In addition, high-throughput comparative genome hybridization arrays may be of interest to confirm alterations found at the genomic level (41).

Critical Issues to Be Considered for DNA Microarrays
Several issues need to be considered when interpreting the results of expression profiling using DNA microarrays in bladder cancer; the importance of these issues depends heavily on the questions the investigator wishes to address and the resources available. These include the type of DNA microarray used; sample preparation, including specimen types, sample availability, and tissue heterogeneity; and the critical area of data analysis.

Issues of cost, set-up time, available personnel, flexibility, and product range will influence the decision of manufacturers or buyers of any of the commercially available oligonucleotide, glass, or nylon cDNA microarrays (58, 59). For experimental design and results interpretation, it is critical to keep in mind that in oligonucleotide chips and nylon macro and radioactive microarrays, the sample under study is directly hybridized to the chip (58). In cDNA glass microarrays, the sample tested is hybridized simultaneously with a reference sample labeled with a different fluorochrome (59).

Critical issues include the control of quantity and quality of the RNA from which the hybridization sample is prepared, especially when the RNA is isolated from clinical specimens. Potential solutions to the quantitative limitations include probe labeling protocols that increase sensitivity through label signal amplification using dendrimers (60), probe amplification protocols that reduce the amount of RNA required through the use of highly efficient phage RNA polymerases or PCR amplification (61, 62), and posthybridization amplification methods in which the target-probe duplexes are detected enzymatically (62). Problems of RNA quality usually are severe when working with blood, urine, or archived samples because many fixing and embedding protocols may damage RNA integrity (63). Nonetheless, the issue of RNA
quality is controversial, at least for certain tumors, when standard fixation protocols are used.

Tumor heterogeneity is a critical issue in tumor expression profiling in general and particularly in the case of bladder cancer. Tumor cells are surrounded by healthy connective tissue and inflammatory infiltrates. In addition to manual microdissection of tissue sections from embedded frozen tumor blocks, laser capture microdissection appears to be a method capable of isolating relatively pure cancer cells populations from clinical specimens (64). However, the extent to which the laser beam affects the quality of the RNA obtained is still controversial. Flow cytometric sorting is also possible, using bladder washes or disaggregated tissue preparations as described previously (49).

Expression profiling experiments produce huge data sets. The outcome of these studies is extremely dependent on the algorithms applied in the analysis. Various mathematical, statistical, and bioinformatic tools have been developed to cluster genes by integrating them with biological pathways or to perform class predictions that identify expression patterns that correlate with phenotypic characteristics, such as tumor type. An international effort is underway to develop guidelines and a consensus on data handling and annotation (MIAME standards), which may facilitate access to the raw data from published microarray experiments in a uniform format.

Final Comments
The aim of this report was to discuss how DNA technologies might provide novel biomarkers for bladder cancer. In the near future, expression profiles or gene clusters may themselves become biomarkers of clinical utility in the management of patients with bladder cancer. Additionally, individual identified molecular targets might be clinically validated and developed into new serum or urinary tumor markers. Because the quality, standardization, and ease of use of the technology are increasing and the costs are decreasing, DNA microarrays may move from being a technology restricted to research or a few well-funded laboratories to clinical laboratories.

References