Clinical Application of cDNA Microarrays in Oncology

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ABSTRACT

DNA microarrays represent an important new tool to analyze human tissues. The technology enables investigators to measure the expression of several thousand mRNA species simultaneously in a biological specimen. This process, called transcriptional profiling, represents a technological breakthrough in the analysis of biological specimens. It may be used to screen for individual genes that are differentially expressed between normal and diseased tissues in the hope of finding novel targets for drug development or finding new single-gene markers of clinical outcome. Microarrays are also applied to learn about the complex biology of cancer by simultaneously monitoring interactions between hundreds of genes during experimental conditions in vitro or during therapy in vivo. Analysis of gene expression patterns may also be used as a classification tool to sort cancer into various clinically relevant subgroups that is not currently possible with other methods. The first clinically important applications of this technology will likely be its use as a tool to refine diagnosis and improve the accuracy of predictions of prognosis and response to therapy. DNA microarrays in several “proof-of-principle” experiments have demonstrated that they can predict important clinical outcomes, including outcomes that cannot currently be predicted with other methods, but the true clinical utility and the limits of this exciting new technology are yet to be established. This paper reviews the current methodology and applications of this technique as they relate to clinical oncology. The Oncologist 2003;8:252-258

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Appreciate how future DNA microarray-based tests will be different from other diagnostic tests.
2. Discuss the potential clinical applications of DNA microarray technology.
3. Define limitations of the technology.

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ABSTRACT

DNA microarrays represent an important new tool to analyze human tissues. The technology enables investigators to measure the expression of several thousand mRNA species simultaneously in a biological specimen. This process, called transcriptional profiling, represents a technological breakthrough in the analysis of biological specimens. It may be used to screen for individual genes that are differentially expressed between normal and diseased tissues in the hope of finding novel targets for drug development or finding new single-gene markers of clinical outcome. Microarrays are also applied to learn about the complex biology of cancer by simultaneously monitoring interactions between hundreds of genes during experimental conditions in vitro or during therapy in vivo. Analysis of gene expression patterns may also be used as a classification tool to sort cancer into various clinically relevant subgroups that is not currently possible with other methods. The first clinically important applications of this technology will likely be its use as a tool to refine diagnosis and improve the accuracy of predictions of prognosis and response to therapy. DNA microarrays in several “proof-of-principle” experiments have demonstrated that they can predict important clinical outcomes, including outcomes that cannot currently be predicted with other methods, but the true clinical utility and the limits of this exciting new technology are yet to be established. This paper reviews the current methodology and applications of this technique as they relate to clinical oncology. The Oncologist 2003;8:252-258

MICROARRAY TECHNOLOGY AND CLINICAL MEDICINE

DNA microarrays represent an important new tool to analyze human tissues. The technology enables investigators to measure the expressions of several thousand mRNAs simultaneously in a biological specimen. The gene density of currently used microarrays ranges from several hundred to over 30,000 unique human sequences [1]. After the almost complete sequencing of the human genome, it has been suggested that the maximum number of human genes is around 30,000-40,000 [2]. The number of genes...
expressed in any particular human cell type is less than that. It is, therefore, technically possible to monitor almost the entire transcriptome, the collection of all mRNAs present, in a tumor specimen. This process, called transcriptional profiling, represents a technological breakthrough in the analysis of human tissues and might impact the practice of medicine as much as the discovery of monoclonal antibodies did. Transcriptional profiling, in general, is used for three overlapping purposes (Table 1). It may be used to screen for individual genes that are differentially expressed between normal and diseased tissues in order to find novel targets for drug development or to find new single-gene markers of clinical outcome. Microarrays can also be applied to learn about the complex biology of cancer by simultaneously monitoring interactions among hundreds of genes during experimental conditions in vitro or during therapy in vivo (Fig. 1). Analysis of gene expression patterns may also be used as a classification tool to sort cancer into various clinically relevant subgroups, which is not currently possible with other methods. Both the identification

<table>
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<th>Table 1. Various applications of DNA microarray technology in medicine</th>
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<td><strong>Screening test to identify potentially important individual molecules such as</strong></td>
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<td><strong>Novel tool to analyze complex cellular behavior</strong></td>
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<td>• To study the transcriptional response of thousands of genes under various experimental conditions or during therapy</td>
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<td>• To identify key molecular pathways involved in the pathogenesis of subsets of cancer</td>
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<td><strong>Classification tool</strong></td>
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<td>• To discover new molecular classes of histologically similar cancers</td>
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<td>• To identify patients prospectively with important clinical outcomes, such as long-term survivors or good responders to a given therapy</td>
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Figure 1. DNA microarray profiles of MCF-7 breast cancer cells after exposure to epirubicin. The images show the complex transcriptional response of MCF-7 cells to epirubicin treatment. Each spot represents a distinct gene, and brighter spots indicate higher gene expression levels. Profiles were obtained at baseline (A) and after 8 (B) and 24 (C) hours of exposure to the drug. Analysis of such data could help to understand which cellular signaling and repair pathways are activated after exposure to a cytotoxic drug and ultimately may lead to strategies to modulate response by selectively interfering with the molecular response that determines whether a cell survives or dies. Arrow head indicates a few of the many genes upregulated by epirubicin.
of novel therapeutic targets and the study of the complex behavior of the transcriptosome are of great importance. However, the first clinically important application of this technology will likely be its use as a tool to refine diagnosis and improve the accuracy of predictions of prognosis and response to therapy.

ANALYSIS OF MICROARRAY DATA

There are two basic approaches to analyzing microarray data. The first approach, often referred to as unsupervised analysis, uses the information provided by all genes that are expressed in a tissue and meet certain quality criteria. The second approach uses only a preselected group of genes to distinguish among a priori defined subsets of cancers and is called the supervised method [3]. Both of these approaches can be applied to the same data set for different purposes. The unsupervised method is less biased and better suited to reveal previously unrecognized tumor subtypes within morphologically similar cancers based on similarities in global gene-expression profiles. However, these molecular subgroups may, or may not, reflect current biologically or clinically important distinctions. The supervised method is better suited to prospectively identify tumors that belong to a predefined clinically important subgroup. There are several mathematical tools to test and display relatedness among gene-expression profiles. These include, among others, hierarchical clustering and multidimensional scaling [4]. The first method yields dendrograms that resemble evolutionary trees, where terminal branches depict closely related specimens. The second generates three-dimensional (3D) scatter plots in which related specimens, represented by dots, cluster together in 3D space. Both of these can be used in supervised or unsupervised analyses. Figure 2 illustrates supervised and unsupervised hierarchical clustering of breast cancer samples. Clustering is an easily accessible way to present and visualize relatedness among gene-expression profiles across several specimens. However, it is not the optimal method to generate predictions about clinical outcome.

Figure 3 illustrates the bioinformatics approach to discovering gene-profile-based outcome predictors. A commonly used strategy for outcome prediction is to select cases with known outcome, for example, tumors that responded and tumors that did not respond to a particular therapy. Next, the

Figure 2. Hierarchical cluster analysis of gene-expression profiles of 36 breast cancer cases. The figure illustrates the relatedness among 36 breast cancers based on gene-expression profiles. Tumor biopsies were taken at diagnosis, before starting preoperative chemotherapy with sequential paclitaxel and FAC. The more similar the expression profiles, the closer the cancers cluster on the terminal branches of the dendrograms. A) The relationship among the tumors when 26,421 unselected genes were used for unsupervised hierarchical clustering. The resulting grouping of tumors does not correspond to pathologic response to preoperative treatment (pCR = pathologic complete response, N = residual disease in the breast or lymph nodes after completion of preoperative chemotherapy). Knowing the clinical outcome after completion of preoperative chemotherapy, it is possible to select genes that are associated with pCR. B) and C) show clustering outcomes when only these highly select groups of genes are used for supervised clustering. Using the top 100 pCR-associated genes, a perfect separation of responders can be seen (B). Using only the top 10 genes, the separation becomes less robust (C).
cases are randomly divided into two groups. The first group (the training set) is used to discover a genomic predictive test, and the second group (the validation set) is used to validate the test and estimate its predictive accuracy in independent cases. The first step is to identify a set of differentially expressed genes between the two outcome groups included in the training set. There are several mathematical tools to accomplish this; one commonly used method is to apply two-sample t-statistics to the gene expression data [5, 6]. Next, these informative genes are combined with machine-learning algorithms (support vector machine, k-nearest neighbor, weighted voting methods, etc.) to generate an outcome predictor test [7, 8]. The definition of a gene-expression profile-based predictive test, therefore, includes a particular class-prediction algorithm and the unique set of genes that are fed into the predictive algorithm to generate a binary “yes or no” classification. Since there are several different algorithms and different numbers of informative genes may be combined with any one of these methods, it is customary to estimate the predictive accuracy of these various classifiers in a leave-n-out cross-validation and random-label permutation tests. The single best classifier is then tested on the independent validation cases to determine its predictive accuracy. Different sets of genes may be selected from the same comprehensive expression profile and fed into different predictive algorithms for different classification purposes. It may be possible to generate prognostic and treatment response predictions simultaneously by applying different predictive tests to the same gene-expression data.

**Improve Diagnostic and Prognostic Accuracy with the Help of cDNA Microarrays**

The histologic diagnoses of several common cancers are relatively straightforward. In many other instances, histologic diagnoses are complex and subjective and the morphological categories are limited in their accuracy to predict clinical outcome. Transcriptional profiling of cancer tissue may assist in the diagnoses of difficult cases and may refine prognostic predictions. There are examples from several laboratories that molecular classification of cancer based on global gene-expression profiles is possible. Unsupervised clustering of breast cancer specimens consistently separated tumors into ER+ and ER− clusters, indicating that ER status has a major impact on the transcriptome of breast cancer [11-13]. Analysis of gene-expression profiles can also distinguish sporadic breast cancers from breast cancer gene, BRCA, mutant cases [14]. Leukemia samples can also be separated into acute lymphocytic or myelogenous
leukemia based on gene-expression profiles [3]. Transcriptional profiles also revealed previously unrecognized molecular subgroups within existing histological categories of cancer. For example, breast cancer may be grouped into luminal or basal epithelial cell types [11]. Diffuse large-B-cell lymphoma may be classified into three distinct molecular subgroups, germinal center B-cell-like, activated B-cell like, or type 3 [15, 16]. Gene-expression-based novel classification schemes have also been proposed for soft tissue and central nervous system embryonal tumors [17, 18]. What makes these attempts clinically relevant is that several investigators also reported distinct clinical outcomes for the different molecular subgroups. Gene-expression profiles have been shown to predict survival of patients with node-negative breast cancer [19, 20], lymphoma [15, 16], renal cell cancer [21], and lung cancer [22]. In the case of breast cancer, gene-expression profile-based outcome prediction appears to outperform existing prognostic classifications [20].

**Prediction of Response to Therapy**

With the exceptions of ER or progesterone receptor expression and HER-2 gene amplification, there are no clinically useful molecular predictors of response to any form of anticancer therapy. The quest for reliable predictive markers remains clinically important. The armamentarium of anticancer drugs has substantially increased in the past decade, however, treatments continue to be applied empirically using a trial-and-error approach. Clinical experience suggests that some tumors are sensitive to several different types of chemotherapeutic agents, particularly at early stages, while other cancers of the same histology show selective sensitivity to certain drugs but resistance to others. Any test that could assist physicians to select the optimal chemotherapy from several alternative empirical treatment options would be an important clinical advance. A large number of biologically targeted drugs have also appeared in the clinical trial arena. Experience with first-generation compounds tested in the clinic suggests that these agents produce modest objective tumor response rates and provide limited clinical benefit in unselected patient populations. The ability to target these interventions to the subset of patients that will benefit from them could be critical for the success of these novel drugs. There are encouraging early results indicating that predictions of response to chemotherapy or biologically targeted agents may be possible by analyzing gene-expression profiles. Investigators identified 95 genes, through microarray experiments, whose expressions could be used to predict sensitivity of acute lymphoblastic leukemia cells to STI571 [23]. An abstract reported that the molecular profiles of breast cancer responding to preoperative docetaxel were different from those not responding [24]. Similarly, gene-expression profiles that may predict complete pathologic response to preoperative sequential paclitaxel and 5-fluorouracil (5-FU), doxorubicin, and cyclophosphamide (FAC) chemotherapy in breast cancer have been identified (Fig. 2) [25]. If these observations are confirmed, this could represent a successful clinical application of microarray technology to a clinical challenge that could not be addressed adequately with previously existing technologies.

**Challenges**

The potential of gene-expression profiling as a novel tool to improve on existing diagnostic, prognostic, and predictive tests is very exciting. However, there are several challenges that need to be addressed before routine clinical application. Some of these relate to the technology itself; others concern clinical utility. In general, there is no consensus or precedent on how to translate observations made through microarray experiments into user-friendly clinical tests. Some believe that a small number of marker genes can be extracted from the profiling results and that these genes can be tested by more conventional techniques, such as immunohistochemistry, in situ hybridization, or PCR, to generate a widely accessible test [26]. Others propose comprehensive profiling of clinical tissues with disease-specific cDNA microarrays and use of the transcriptional profiling itself as the clinical test [27]. A very important challenge is standardization. Currently, multiple microarray platforms exist that use distinct sets of genes and employ different hybridization and signal-detection methods. Some arrays contain cDNAs of variable lengths; others contain small oligonucleotide sequences. The same gene may be represented by different sequences in different oligonucleotide arrays, which could result in differences in signal intensity. Investigators who utilize competitive hybridization between fluorescein-labeled clinical samples and control samples invariably use different controls from laboratory to laboratory. Not surprisingly, marker sets generated by one group often differ substantially from marker sets generated by others for the same purpose. All current transcriptional profiling methods require fresh or frozen tissue, and the type of tissue-sampling method may influence the profiling results. Transcriptional profiles are a composite of mRNA contributed by all tissue components of the sample. Fine needle aspirations, core needle biopsies, and surgically resected specimens yield somewhat different transcriptional profiles from the same tumor [28]. The interpretation of microarray results is also substantially different from the interpretation of conventional molecular diagnostic tests. For example, immunohistochemistry to determine ER expression can be performed using different antibodies that all recognize the
ER protein, and trained pathologists can interpret the staining in a reasonably similar way. In contrast, the interpretation of a test whose final result depends on the constellation of several dozen to several hundreds of different genes requires specialized computer software to run a classification algorithm. Furthermore, the predictive precision of such a test may change as more and more clinical outcome data become available. This would necessitate periodic revisions to better fit prediction with observed clinical outcomes.

Another important challenge that cDNA microarray-based tests, like all other potential diagnostic tests, will face is to demonstrate clinical utility. How accurate does a prognostic or predictive test need to be in order to become clinically useful? The answer may depend on the clinical circumstances [29]. For example, combined-modality adjuvant systemic therapy may result in an annual reduction of risk of recurrence by as much as 50% in patients with newly diagnosed breast cancer [30]. In contrast, the same treatment fails to cure patients with macroscopic metastatic disease. Therefore, women diagnosed with breast cancer face a difficult and complex decision at the time of diagnosis. They may be cured by surgery alone, or they may harbor micrometastatic disease. A subset of patients who harbor micrometastases will be cured by systemic adjuvant chemotherapy and/or hormonal treatment. If they miss this opportunity to maximize their chance of cure and the cancer recurs, cure is no longer attainable. It has been reported that, in this situation, a substantial number of women in the U.S. are willing to undergo adjuvant chemotherapy to gain a 2%-5% absolute improvement in survival [31]. Indeed, current recommendations call for considering adjuvant chemotherapy for most breast cancers >1 cm in size, even though the long-term risk of relapse from small, lymph-node-negative tumors may not be greater than 10%-20% [32, 33]. Under these circumstances, a prognostic test that aims to identify women who are cured by surgery alone, and therefore do not need any further systemic therapy, will need to show a very high degree of predictive precision. A potentially lifesaving treatment should not be denied to a patient mistakenly classified into the low-risk group.

In other clinical situations, a prognostic or predictive test of lesser precision may be acceptable. For example, there are a number of commonly used adjuvant chemotherapy regimens for breast cancer including combinations of 5-FU, doxorubicin, and cyclophosphamide (FAC, CAF, AC), cyclophosphamide, methotrexate, and 5-FU (CMF), or newer regimens including taxanes (docetaxel [AC] and paclitaxel [FAC]). A predictive test that aims to select the optimal standard adjuvant chemotherapy from several empirically used regimens may not need to be perfect in order to be clinically useful. For example, if one could predict that an individual had a 50%-60% chance of complete pathologic response to a certain type of preoperative chemotherapy for breast cancer, compared to the average chance of 25%-30% with the best unselected regimens, this could be considered an important clinical advance over the current empirical use of chemotherapy. One can foresee extended phase II clinical trials of novel agents where microarray profiling (in the absence of single-gene predictors of response) is used to identify patients who benefit from the new drug. Definitive phase III trials could then be conducted with relatively modest sample sizes (but with large screening populations) to demonstrate important benefits for those prospectively selected patients. It is likely that such applications will be among the first clinical uses of transcriptional profiling. DNA microarrays in several proof-of-principle experiments have demonstrated that they can predict important clinical outcomes, including outcomes that cannot currently be predicted with other methods, but the true clinical utility and the limits of this exciting new technology are yet to be established.

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Available at: http://consensus.nih.gov/cons/114/114_intro.htm