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Application Note

Microarrays in Cancer Research: Recent Advances and Future Directions

An important goal in cancer research is to identify significant genomic alterations responsible for the emergence and progression of disease. It is now possible, with Affymetrix® brand products, to perform extensive analysis of tumor genomes, including whole-genome chromosomal copy number analysis, systematic gene resequencing, and RNA expression analysis. This application note is to review recent advances in cancer research, how Affymetrix products have been applied in cancer research, and to highlight future directions enabled by new GeneChip® microarray applications for DNA methylation, splice variation, and targeted genotyping analysis.

Introduction

Cancer is a highly complex disease which can encompass multiple genomic alterations, including point mutations, translocations, gene amplifications, epigenetic modifications, deletions, aberrant splicing, and altered gene expression. These changes may be inherited or somatically acquired during progression from a normal to a cancerous cell. During the past decade, increasingly research results have distinguished how these genomic perturbations drive cancer cell survival by altering the mechanism for cell cycle control, DNA repair, differentiation, apoptosis, tumor vascularization, and metabolism.

By improving the understanding of these molecular mechanisms, scientists have gained greater insight into the initiation of cancer, its progression, and its sensitivity to therapeutics. The goal of cancer research is ultimately to improve the diagnosis and the treatment of cancer through more accurate disease classification and patient stratification, which allows for the design of therapies that are more targeted to specific cancer subtypes and potentially improves the effectiveness of existing regimens based on therapeutic response and adverse events (Figure 1).

This application note reviews examples of cancer research using Affymetrix products, spanning from discovery research to validation and into clinical utility (shown in Figure 1), including:

- Identification of cancer biomarkers and therapeutic targets
- Elucidation of the mechanisms of cancer pathways
- Validation of therapeutic targets and cancer biomarkers
- Clinical classification and stratification

Figure 1: Cancer Research Workflow.

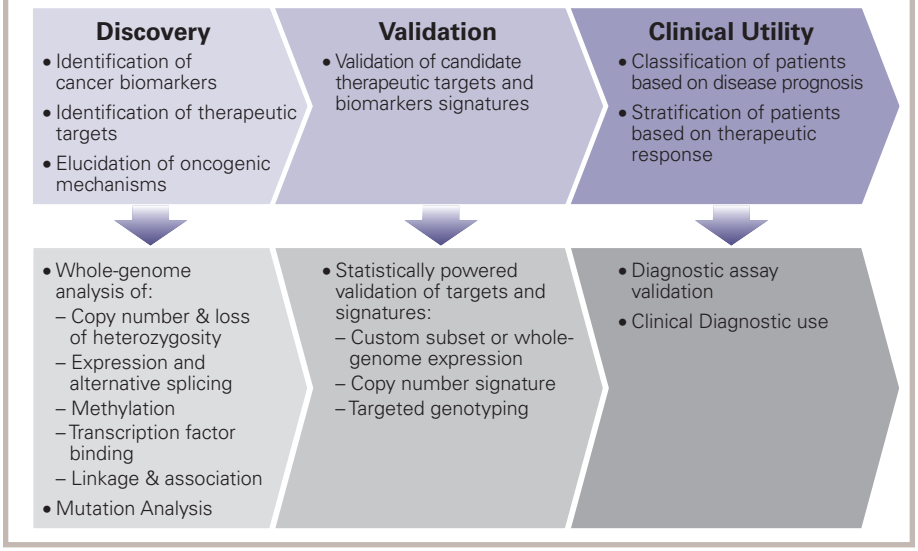


Table 1: Detecting genetic events in human tumors with Affymetrix products.

Genetic Events	Common Detection Methods	Applicable Affymetrix GeneChip® Products/Services
Genetic Susceptibility	Linkage & Association	– Mapping Arrays
Somatic and germ line mutations	Resequencing of candidate genes	– CustomSeq® Resequencing Arrays
	SNP Identification and genotyping	– Custom Targeted Genotyping SNP Kits – Application Specific Targeted Genotyping SNP Kits – MRD for SNP discovery
Gene amplification and deletion	Comparative genome hybridization	– Mapping Arrays
	Gene- and exon-level amplification analysis	– Exon Arrays – Whole-Genome Tiling Arrays
Epigenetic modification	Methlation PCR/bisulfite sequencing, ChIP	– Whole-Genome Tiling Arrays – Promoter Tiling Arrays
Altered gene expression level	Gene-and exon-level expression	– 3' Expression Arrays – Exon Array, Promoter Tiling Arrays – Whole-Genome Tiling Arrays
Alternative splicing	Exon-level expression	– Exon Arrays
Gene Regulation	ChIP	– Whole-Genome Tiling Arrays – Promoter Tiling Arrays

Discovery

TARGET/BIOMARKER DISCOVERY AND DISEASE MECHANISM ANALYSIS

A comprehensive characterization of all of the genetic, genomic, and epigenetic modifications associated with the cancer is critical for the understanding of the origins of tumor process, and for finding the targets of therapeutic interventions. Utilization of high-density nucleic acid microarrays is one of the most effective approaches to identifying these key molecular events (Table 1), and increasingly, researchers are combining multiple genome-wide approaches to further enhance their understanding of pathway effects.

A. Whole-genome expression profiling reveals novel molecular pathways and markers for cancer progression.

Over the past decade, global gene expression profiling has become a standard tool for pathway and biomarker discovery. In the past 15 years, nearly 1,000 peer-reviewed publications attest to the value of GeneChip® microarray technology in the area of oncology and hematology.

In just one recent example, Kimchi, *et al.* used the GeneChip® Human Genome U133A Array to uncover a novel pathway involved in esophageal cancer pathogenesis.[1] Evaluation of matched samples of normal esophageal epithelium, Barrett's metaplasia, and esophageal adenocarcinoma revealed a 214 gene signature that distinguishes across the three sample types.

Their data indicate that transformation of Barrett's esophagus to adenocarcinoma is associated with suppression of the genes corresponding to the epidermal differentiation complex. Correlation analysis of genes concordantly expressed in Barrett's esophagus and adenocarcinoma further revealed 21 genes that represent potential genetic markers of disease progression and pharmacologic targets for treatment intervention.

B. Copy number and loss of heterozygosity (LOH) analysis

Oncogene amplification and/or the deletion of tumor suppressor genes are hallmarks of cancer initiation and progression and have recently been implicated in response to therapeutic agents. Comparative Genome Hybridization (CGH) and loss of heterozygosity (LOH) analysis are standard approaches used to characterize copy number changes on a whole-genome or candidate gene level. Traditional techniques, such as microsatellite analysis and BAC CGH, are laborious, have limited whole-genome resolution, and represent challenges to data standardization initiatives.

GeneChip® oligonucleotide arrays provide a standard platform to enable data standardization, as well as higher resolution, to detect smaller changes and map genetic boundaries. Furthermore, single nucleotide polymorphism (SNP) arrays offer significant advantages over traditional methods by combining copy number, LOH, and SNP genotyping analysis into a single assay, thereby enabling the detection of copy neutral LOH events. Coupling this analysis with gene expression profiling provides complementary insight into pathway impacts of such genomic changes, further accelerating gene discovery.

Garraway and colleagues[2] demonstrate the power of combining copy number and expression information. Using the GeneChip® Human Mapping 100K Set, this group scanned more than 100,000 genetic variations from 58 cancer samples, identifying copy number aberrations in microphtalmia-associated transcription factor (MITF), where MITF amplification was correlated with decreased survival (see Figure 2).

Using the SNP arrays, they found a 3.5 megabase region of the genome – containing approximately 14 genes – that was duplicated in melanoma samples. They honed in on the MITF genes by analyzing GeneChip® Human Genome U95 Array (HG-U95) expression data for over 12,000 genes, noticing that only one of the 14 duplicated genes was highly expressed in melanoma.

Figure 3 illustrates the increased MITF expression associated with chromosome 3p amplification in melanoma cell lines. Melanoma patients with extra copies of MITF showed a decrease in five-year survival. In contrast, chemotherapy drugs killed melanoma cells more effectively if MITF protein activity was reduced in these cells.

C. Genome-wide analysis of epigenetic modifications and impacts on gene expression

Epigenetic modifications, such as DNA and histone methylation, and histone acetylation, are a rapidly growing focus for cancer researchers. In humans, gene methylation is known to regulate the expression of up to 60 percent of genes.

Utilizing whole-genome expression products, researchers have leveraged chemical demethylation to globally analyze the impact of genes silenced in cancer tissues and cell lines. Lodygin, *et al*. utilized the HG-U133A oligonucleotide arrays to simultaneously study the methylation impact in prostate cancer cell lines.[3] Out of several hundred genes that were induced through chemical demethylation with 5-aza-2'-deoxycytidine and trichostatin A, fifty were linked to known tumor suppressor activity.

Through methylation-specific PCR analysis within these cell lines and 41 primary prostate cancer samples, six genes out of the fifty showed consistent promoter CpG methylation and gene silencing that was absent in normal prostate tissue. Based on this study and others that suggest gene silencing is primarily important in early-stage tumor development, CpG methylation of these six genes has potential diagnostic applications for early disease diagnosis.

Today, GeneChip® promoter and tiling arrays provide a new methodology for genome-wide analysis of epigenetic modifications through chromatin immunoprecipitation (ChIP)-on-chip experiments. In the January 2005 issue of *Cell*, Bernstein, *et al*. utilized ChIP-on-chip to map histone H3

Figure 2: Garraway L.A., *et al*. Integration of expression and copy number data facilitates candidate gene discovery. Schematic of the integrated genomic approach – Reprinted by permission from McMillan Publishers Ltd: *Nature* **436:** 117-22 (2005).

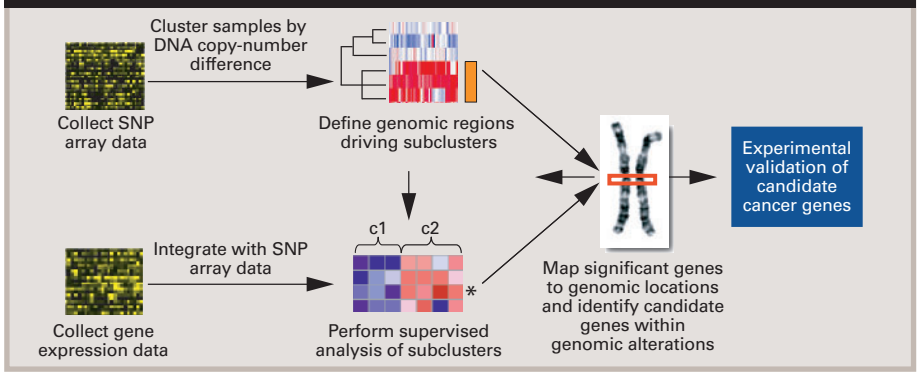
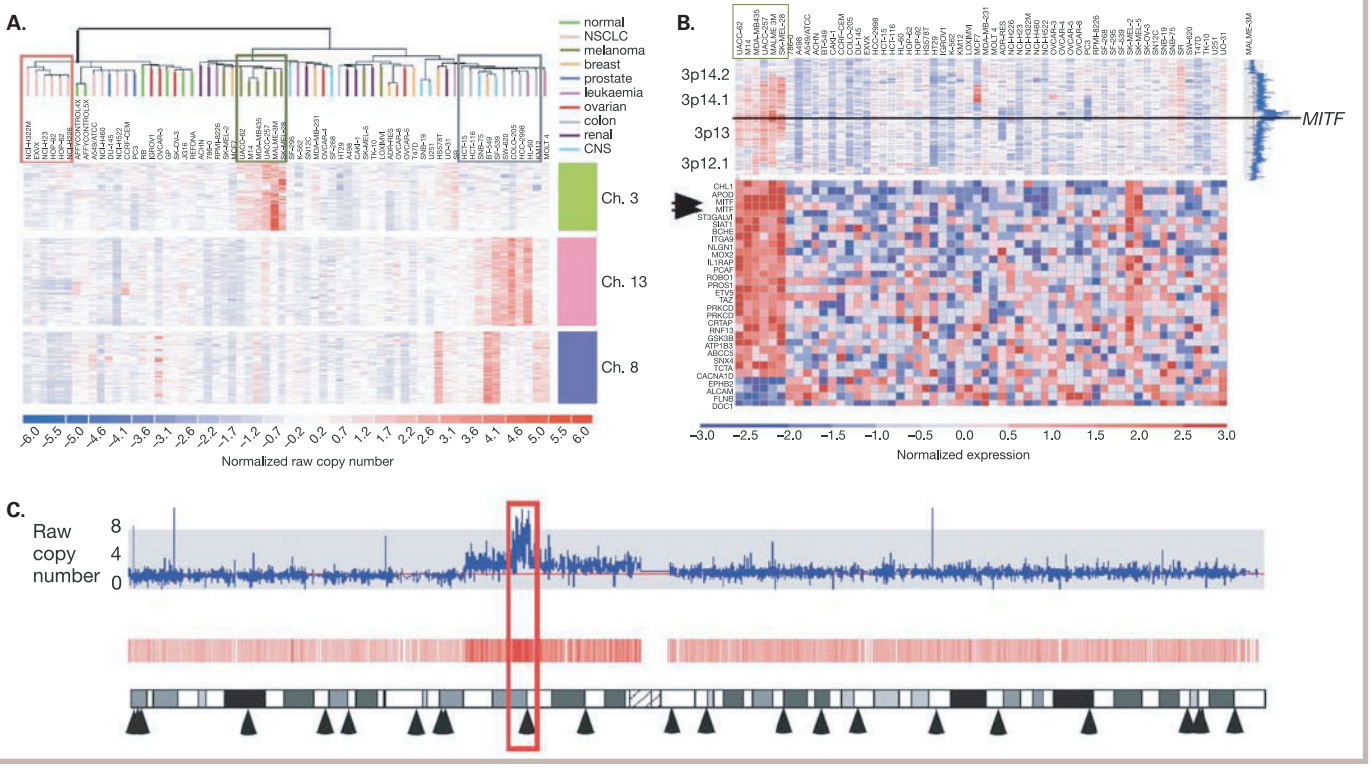


Figure 3: Garraway L.A., *et al*. Increased MITF expression associated with chromosome 3p amplification in melanoma cell lines. **A.** Hierarchical clustering of raw copy number data **B.** Integration of copy number and gene expression data **C.** Chromosome 3 copy number, SNP signal intensity (middle) and cytoband map. – Reprinted by permission from McMillan Publishers Ltd: *Nature* **436:** 117-22 (2005).



lysine 4 di- and trimethylation, and lysine 9/14 acetylation across nonrepetitive portions of human chromosome 21 and 22.[4] In addition, Bernstein mapped six orthologous genomic loci in mouse and human, encompassing the cytokine cluster, IL-4 receptor region, and four Hox clusters. Combined, the resulting maps represent more than 39 million base pairs of genome – over three orders of magnitude more coverage than prior studies.

Their results showed distinct regions of histone trimethylation across chromosomes 21 and 22 that correlated well to the 5' start site of annotated genes, and that genes with such histone trimethylation were significantly more active than genes without such histone methylation. Regions that were primarily histone dimethylated were associated near or within introns of highly expressed genes, however were not clear predictors of start sites. Furthermore, comparison of mouse and human loci showed that histone methylation patterns across orthologous loci are conserved, even when the underlying sequence is not, and that broad-based methylation may be important in maintaining Hox gene expression across both species. These studies are demonstrating the importance of studying epigenetic alterations in cancer.

D. *Genome-wide survey of transcription factor binding sites*

Many known oncogenes are transcription factors. However, understanding the networks regulated by these factors or their mutant counterparts on a genome-wide scale have been challenging. It is very important to understand the downstream effects of the mutations within these transcription factors in order to decipher disease mechanisms.

One example of the usage of GeneChip® arrays for such a genome-wide approach is in research performed on estrogen regulation and cancer. Estrogen plays essential roles in endometrial cancer and in breast cancer. With the completion of the human genome, the expressed regions of protein-coding genes for estrogen have been identified, but a great deal remains to be learned about estrogen's cis-regulatory elements.

Using the powerful combination of chromatin immunoprecipitation (ChIP) and tiled oligonucleotide arrays to study protein-DNA interactions across human chromosomes 21 and 22, Carroll, *et al*. identified a number of distal estrogen receptor (ER) binding sites involved with transcriptional regulation that were previously not known.[5] Additionally, the team found that the Forkhead protein FoxA1 is required at these sites for ER activity. Knockdown of the Forkhead protein FoxA1 blocked the expression of ER with chromatin and estrogen-induced gene expression, thus showing that FoxA1 mediates estrogen response in breast cancer cells. By using ChIP and GeneChip Tiling Arrays, Dr. Carroll and his team were able to identify chromatin regions, both proximal and distal to promoters, which are involved in mediating ER transcriptional activity. Finding the Forkhead binding motifs and identifying the functional role of FoxA1 in estrogen signaling demonstrates the power of this approach for identifying critical regulatory regions within previously unexplored sequences of the human genome.

E. *Somatic and germline mutation analysis of candidate genes*

Analysis of accumulating mutations in tumor cells provides insight into the mechanism of disease progression and potentially into therapeutic resistance. Comparison of genetic variation between matched tumor and normal samples may be done on a whole-genome level, as in LOH and copy number analysis, or by using a targeted candidate gene approach. Using GeneChip CustomSeq® Resequencing Arrays to search for mutations in a set of 164 exons from genes associated with cancer, Tengs, *et al*. found both somatic and germline mutations in non-small cell lung cancer (NSCLC).[6] In particular, they found two mutations in the MET proto-oncogene. One mutation, the T10101, was present in a small fraction of lung tumors and may represent a true cancer-causing genotype. The other mutation (N375S) seems to be a common germline polymorphism. The overall performance of the platform was comparable to Sanger-based sequencing, suggesting that microarrays are feasible alternative technology for gene mutation detection and discovery.

FUTURE DIRECTIONS

A. *Combining multiple microarray-based applications*

Increasingly, researchers are proving the value of combining more than one microarray-based application to increase the understanding of cancer pathways. As noted earlier, Garraway, *et al*. demonstrated that genome-wide copy number and gene expression analysis are complementary methods that can guide candidate gene discovery.[2]

In results to be published, researchers led by Tim Triche at Los Angeles Children's Hospital integrated gene expression and splice variant analysis with copy number information to provide a more complete picture of Embryonal and Alveolar Rhabdomyosarcoma (RMS). This type of holistic approach led to identification of genomic signatures that more accurately distinguished these two subsets of RMS, and that are also associated with prognosis. The discoveries also suggested novel therapeutic approaches using siRNA that are currently under evaluation. A preview of this work can be found in the *Spring 2006 Affymetrix Microarray Bulletin*, where the workflow is outlined in detail.[7]

B. *Alternative Splicing*

Alternative splicing plays an important role in regulation of development and differentiation and is hypothesized to result from a dynamic interaction between trans-acting factors that bind to regulatory elements in the pre-messenger RNA.[8] While it increases protein diversity by allowing multiple functionally distinct proteins to be coded by the same gene, RNA splicing can become aberrant, as frequently seen in human cancer cells.[9,10]

Combining a novel array design strategy with the highest-density array manufacturing capability, GeneChip® Exon Arrays provide, for the first time, exon-level expression profiling at a whole-genome scale on a single array.[11] The GeneChip® Exon Array system offers new dimensions for researchers to explore global expression of individual exons to uncover and document novel alternative splicing, alternative promoter use, and alternative termination events. It combines genome-wide exon-level expression data with sequence information to reveal the phenotypic splicing patterns resulting from genetic variations.

In the work led by Triche described above,[7] alternative splicing of the PAX 7 and arginosuccinate synthetase genes were shown to distinguish between the two subtypes of Rhabdomyosarcoma – alveolar (ARMS) and embryonal (ERMS). In both cases, exon-level expression uncovered previously unknown alternative splicing activity within these genes.

Figure 4: Dave S.S., *et al.*[17] Survival associated with prognosis in Follicular Lymphoma. Panel A: overview of survival signature analysis and approach used for the development and validation of a survival predictor based on gene expression. Panel B shows a Kaplan-Meier survival curve for all the patients for whom these data were available.
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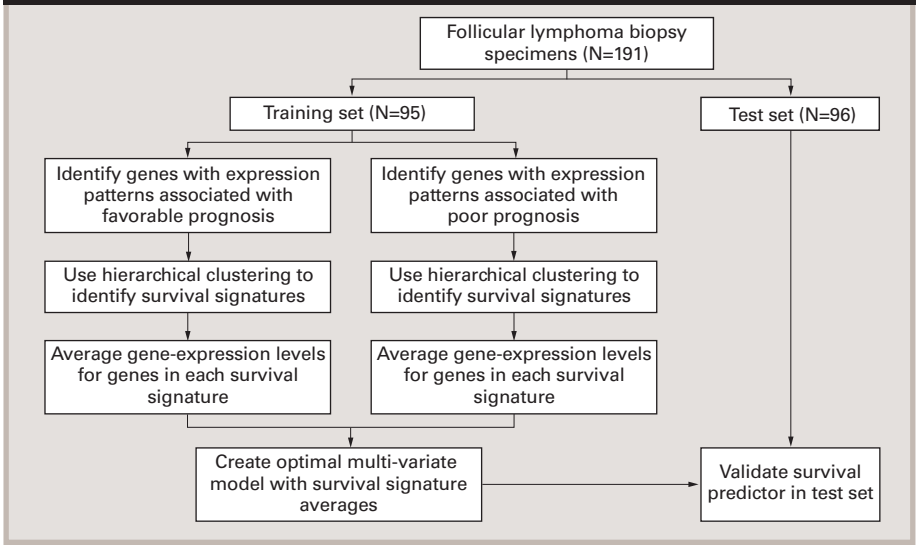


C. Unbiased discovery of novel transcripts

While GeneChip® 3' Expression and Exon arrays quantify the levels of known and annotated transcripts, new GeneChip® Tiling Arrays are designed to interrogate genomes at regular intervals, including both annotated and so-called "junk" regions of a genome, without bias. Tiling Arrays can be used as a discovery tool to identify novel transcriptional elements and evaluate antisense transcription.[12]

Using this neutral approach to array design, Tiling Arrays have been used in transcriptome studies to discover novel transcripts of unknown function, or TUFs. In work led by Tom Gingeras at Affymetrix Research Laboratories and various collaborators, thousands of TUFs have been identified, many of which are expressed in a coordinated fashion with coding transcripts and that share many properties of coding genes, including cell type expression specificity, nuclear processing and transport, and cellular localization.[13] These findings imply an increasingly complex role for regulatory RNAs that transcend its function within canonical gene transcription and translation.

D. Somatic and germline mutation analysis of candidate genes

Application-specific and custom assay kits using the Molecular Inversion Probe (MIP) assay are also available for mutation analysis of candidate genes. The flexibility and high conversion rate of the MIP technology (typically >90%) allows customers to develop custom SNP panels targeting their specific genes of interest. Application specific assays include a Human Cancer SNP panel that provides comprehensive representation of SNPs in genes believed to be important in cancer.

E. Analysis of formalin-fixed, paraffin-embedded (FFPE) samples

Routine tissue processing has generated banks of formalin-fixed, paraffin-embedded (FFPE) tissue that could be used in retrospective studies. Generating quality genetic data from FFPE samples is challenging due to differences in fixation and extraction protocols, as well as the age of the sample. Thompson, *et al*. demonstrate the potential to use GeneChip® Mapping 10K Arrays for copy number analysis using reasonable quality DNA isolated from FFPE samples[14], and several ongoing studies are investigating the performance of various FFPE protocols on higher resolution 500K arrays for copy number analysis.

F. Genetic association

The GeneChip® Human Mapping 500K Array Set is the third-generation product in the mapping portfolio. It also uses the whole-genome sampling analysis (WGSA) assay and genotypes more than 500,000 genotypes in a single experiment, enabling researchers to conduct highly powered genome-wide studies pertaining to disease genetics, drug response, and linkage disequilibrium. This increase in the number of SNPs allows for higher density, genome-wide mapping sets, which in turn will increase the amount of information that can be extracted for association studies, the identification of DNA copy number alterations, and population substructure.[15] For greater in depth studies the GeneChip® Human Mapping 500K Array may be combined with an Application-specific SNP Kit (using the Molecular Inversion Probe assay) that further extends the genomic coverage.

Validation

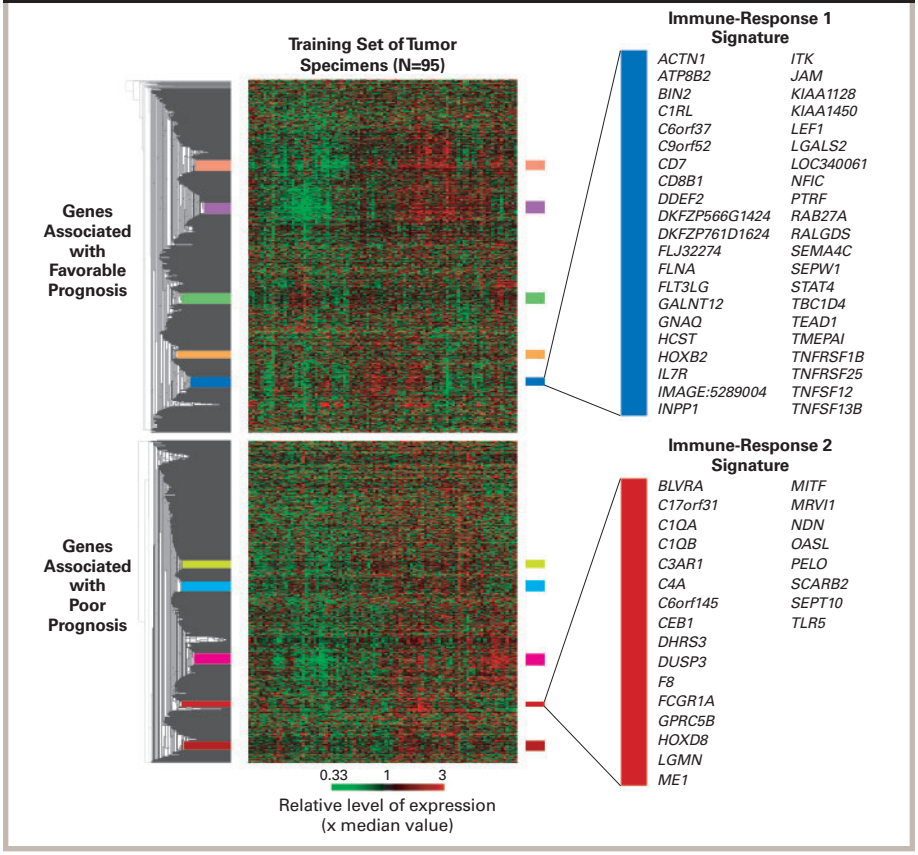
TARGET/BIOMARKER VALIDATION

Microarray expression profiling has been invaluable in identifying putative classifiers for patient prognosis and tailoring therapeutic regimens. However, translating these genomic changes into robust tools with broad clinical utility has been difficult. To date, uptake of biomarker signatures has been limited by insufficiently powerful validation of these signatures. Study design plays a crucial role in providing the correct strength for validation. Richard Simon highlights several critical factors in developing and validating therapeutically relevant genomic classifiers[16], including algorithm selection, feature selection for the classifier (e.g., inclusion of which genes), and proper internal validation (split-sample validation or cross validation).

In a 2004 *New England Journal of Medicine* article, Dave, *et al*. demonstrated one approach to validating prognostic signatures for survival in patients with Follicular Lymphoma using the HG-U133A and B Arrays.[17] In this study, whole-genome expression profiling was performed on 191 biopsy specimens, split between a training and validation set (see Figure 4). Based on the 95 sample training set, hierarchical clustering was performed to identify signatures associated with good and poor prognosis (see Figure 5). Two emergent signatures, when combined, proved highly predictive of four disparate median lengths of survival.

Another critical element in signature and assay validation is reproducibility. Dobbin, *et al*. performed an inter-laboratory comparability study of cancer gene expression analysis using GeneChip® Human Genome U133A Arrays. They found high within- and between-laboratory correlations on their purified RNA samples, cell lines, and frozen tumor tissues.[18] Their findings indicate that, under well-controlled conditions, it is feasible to perform complete and consistent tumor microarray analysis, from tissue processing to hybridization and scanning, at multiple independent laboratories for a single study.

Figure 5: Dave S.S., *et al*.[17] Genes associated with prognosis in follicular lymphoma. – Reprinted by permission from Massachusetts Medical Society: *N Engl J Med* 2004; **351**:2159-69



Addressing factors of study design and reproducibility will provide confidence in the quality of the validated signature, whether it is performed on Affymetrix whole-genome or targeted custom arrays.

FUTURE DIRECTIONS

Custom expression, genotyping, and resequencing

In validating biomarker signatures, many researchers rely on extending whole-genome analysis to larger patient subsets. However, many researchers wish to narrow follow-up validation work to subset genes in order to focus on only their putative regions of interest in a cost-effective manner. The flexibility of the GeneChip® platform is amenable to both approaches for expression, genotyping, and resequencing applications.

The MIP-based Targeted Genotyping (TG) assays for custom and application-specific genotyping enable scientists to conduct validation studies focused on SNPs identified in whole-genome association or copy number studies.[19] The MIP-based assay addresses the challenges of a targeted approach by offering flexible multiplexing from 1,500 to 50,000 SNPs in a single assay. Additionally, the same chemistry is involved across all of these levels of multiplexing, therefore avoiding the need for multiple experiments or major changes in protocols or workflow.

The GeneChip CustomSeq® Resequencing Array program enables analysis of up to 300KB of unique, high-quality, double-stranded sequence on a single array. Custom arrays can be designed to cover contiguous regions, multiple dispersed fragments or genes, providing a cost-effective and efficient method for large-scale resequencing of candidate genes.

The GeneChip CustomExpress® Array

Program allows researchers to select from multiple array formats to accommodate from 520 to 61,200 gene sequences per array.

Clinical Utility

CLINICAL STRATIFICATION/THERAPEUTIC RESPONSE

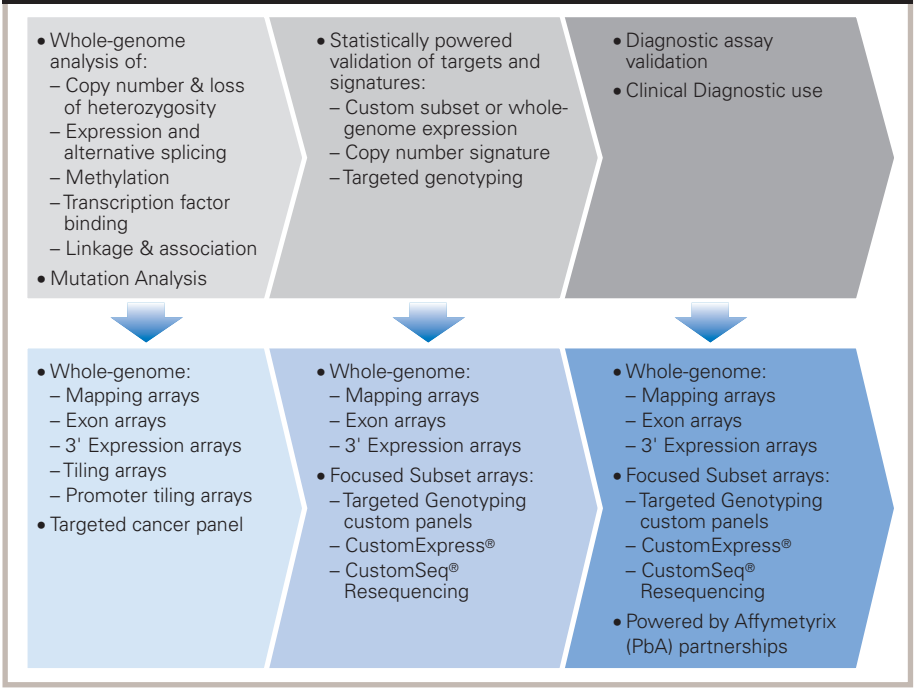
In 2004, the tumor analysis best practices working group stated that "microarrays—in particular, Affymetrix oligonucleotide arrays—are becoming increasingly important in human clinical trials, both for differential diagnosis and monitoring of pharmacological efficacy".[20] Since then, results from over 15 projects involving Affymetrix microarray analyses in various phases of drug development in preclinical or clinical trials have been published.

In a recent Phase III clinical trial Novartis Pharmaceuticals used expression profiles to predict the success or failure of Gleevec® treatment on chronic myelogenous leukemia.[21] They analyzed gene expression patterns from patients prior to treatment and found a 31-gene "No Response" signature, which predicts a 200-fold higher probability of failed therapy.

Similarly, in a Phase II clinical trial conducted at the Dana Farber Cancer Research Institute for the Millennium Pharmaceuticals drug Velcade®, researchers used GeneChip® expression arrays to collect pharmacogenomic data from myeloma patients treated with the drug, identifying candidate gene markers that predict response.[22]

In addition to supporting clinical evaluation of novel therapeutics, microarray analysis has proven valuable in demonstrating efficacy among established cancer therapeutics. Holleman, *et al*. tested leukemia cells from 173 children with acute lymphoblastic leukemia (ALL) for sensitivity *in vitro* to prednisolone, vincristine, asparaginase, and daunorubicin.[23] Using the GeneChip® Human Genome U133 Array, they discovered a set of 124 genes that are associated with sensitivity or resistance to these therapeutic agents. A combined gene-expression score of resistance to the four drugs, as compared with sensitivity to the four, was significantly and independently related to treatment outcome in a multivariate analysis. Of the 124 genes identified, 121 had not previously been associated with resistance to the four drugs they tested. Their findings could help physicians suggest alternate courses of more effective treatment or avoid unnecessary side effects of an ineffective drug.

Figure 6: From target/biomarker discovery, to target validation and clinical application: relevant Affymetrix GeneChip® product families in cancer research.



PATIENT STRATIFICATION AND PROGNOSIS

Genomic changes can help define markers that can predict the patient prognosis and pharmaceutical response.

As first highlighted above, Dave and colleagues utilized GeneChip® HG-U133A and B DNA microarrays to develop a molecular predictor of the length of survival for patients with follicular lymphoma who were either under observation, receiving standard chemotherapy regimens, or receiving autologous stem cell transplantation.[17] A molecular predictor of risk was constructed in a preliminary group of 95 patients and was then tested in a validation group of 96 patients. The accuracy of this predictor was compared with that of the international prognostic index. Four prognostic classes were defined based on gene expression risk score. Upon further analysis the length of survival among these patients correlated with the molecular features of nonmalignant immune cells present in the tumor at diagnosis.

Conclusion

Affymetrix offers a broad portfolio of solutions for cancer research on a single industry-standard microarray platform (see Figure 6). The various examples cited in this application note have shown Affymetrix GeneChip® microarrays to be invaluable across the spectrum of cancer research to date. Future directions involve providing even greater breadth and depth of analysis of the cancer genome – by enabling previously unavailable views of alternative splicing and gene regulation, and by enabling deeper views through higher density tiling for unbiased transcript mapping, and higher density SNP and copy number analysis.

In addition, researchers are increasingly leveraging the true breadth of the platform by combining multiple microarray-based applications to concurrently evaluate different genomic phenomena.

In providing the only microarray instrument platform* that is FDA-approved and CE marked in the European Union for in vitro diagnostic use, Affymetrix provides a pathway for clinical laboratories and diagnostic partners to advance signatures to the clinic. Today, over 15 oncology assays are under development on this platform, encompassing screening through prognosis and monitoring.

Through the use of these tools, researchers can achieve a more complete picture of the genesis and evolution of cancer, with the goal of ultimately improving the diagnosis and treatment of the disease.

*Microarray instrument platform includes the GeneChip® Scanner 3000 Dx, AutoLoader Dx, Fluidic Station 450 Dx and the GeneChip® Operating Software Dx.

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