Workshop on Cancer Biometrics: Identifying Biomarkers and Surrogates of Cancer in Patients

A Meeting Held at the Masur Auditorium, National Institutes of Health

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Summary: The current excitement about molecular targeted therapies has driven much of the recent dialog in cancer diagnosis and treatment. Particularly in the biologic therapy of cancer, identifiable antigenic T-cell targets restricted by MHC molecules and the related novel stress molecules such as MICA/B and Letal allow a degree of precision previously unknown in cancer therapy. We have previously held workshops on immunologic monitoring and angiogenesis monitoring. This workshop was designed to discuss the state of the art in identification of biomarkers and surrogates of tumor in patients with cancer, with particular emphasis on assays within the blood and tumor. We distinguish this from immunologic monitoring in the sense that it is primarily a measure of the tumor burden as opposed to the immune response to it. Recommendations for intensive investigation and targeted funding to enable such strategies were developed in seven areas: genomic analysis; detection of molecular markers in peripheral blood and lymph node by tumor capture and RT-PCR; serum, plasma, and tumor proteomics; immune polymorphisms; high content screening using flow and imaging cytometry; immunohistochemistry and tissue microarrays; and assessment of immune infiltrate and necrosis in tumors. Concrete recommendations for current application and enabling further development in cancer biometrics are summarized. This will allow a more informed, rapid, and accurate assessment of novel cancer therapies.

Key Words: immune response, immune therapy, cancer, biomarkers, surrogates, microarrays, genomics, proteomics, transcription analysis, genetic polymorphism, cytokines, high-content screen, immunohistochemistry, immune infiltration, tumor necrosis, biologic therapy, cancer biometrics

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Cancer is a systemic disease characterized by disordered cell death associated with blockade of apoptotic pathways and secondary, reparative cell proliferation due to genetic and epigenetic changes that affect control of cell death, cell growth, and differentiation. From oncogenesis to metastatic disease, the malignant transformation results from the accumulation of genetic and epigenetic changes that leads to an unstable and dynamic state. Although the intrinsic genomic instability of cancer is primarily driven by characteristics of the cancer cells, it is also influenced by host processes. For this reason, cancer is a disease whose complexity can be appreciated and understood only by using multiple approaches. Identification of high-risk populations likely to relapse after removal of minimal disease could allow implementation of therapeutic strategies at an early stage (prognostic markers). In addition, the identification of easily accessible genetic biomarkers representative of tumor presence and extent prior to a radiologically or clinically detectable size may allow direct clinical follow-up of clinical effectiveness (surrogate markers for therapeutic endpoints). Thus, the effectiveness of novel biologic therapies could be followed in real time without

relying solely on the classic survival or objective response benefit endpoints that, although unarguably the ultimate goal, may not represent an effective screening tool for identification of promising therapies.

So, then, what do we designate as "biomarkers" or "surrogate of tumor" in patients?

Biomarkers are biologic molecules that are indicators of a physiologic state and hallmarks of change during a disease process. A biomarker provides early indication of disease or monitors the progression of the disease. For practical purposes, a biomarker should be easy to detect and measurable across diverse genetic populations. In addition, a biomarker should be amenable for use in the detection of cancer at an early stage, for the identification of high-risk individuals, for early detection of recurrence, or as an intermediate endpoint in assessment of chemoprevention. "Surrogate markers," also called surrogate endpoints, become relevant when the clinical endpoint of primary interest is difficult to follow. With particular reference to cancer proteomics, it may not be easy, for example, to detect a single protein as a specific tumor biomarker. As "alternative/surrogate" marker, a "pattern" of physiologic events may be selected as a more suitable indicator of a disease or its progression. Gene mutations, alterations in gene transcription and/or translation, and alterations in the resulting protein products can all potentially be used as specific biomarkers for disease and cancer.

Searching for prognostic or surrogate markers might be most effective if high-throughput methods for approaching the dynamic and multidimensional aspects of cancer could be globally implemented. It is likely that the study of cancer biology and the outcome of biologic therapy will be best pursued at multiple and complex levels that could efficiently encompass the genetic complexity of cancer and its host. Functional genomic approaches offer the unprecedented opportunity to perform such studies on a large and comprehensive scale. This strategy shifts cancer research from a paradigmatic hypothesis-driven approach to a hypothesis-generation approach in which high-content nets are used to sieve unknown information resting below the surface of our knowledge. Discoveries using this approach have already expanded our understanding of cancer biology, progression, and response to biologic therapy.

The evolution of biomarker research has progressed from histologic/blood-based plasma or serum markers (first generation) to the search for mutation-based markers (second generation), genomic-based markers (third generation), and proteomics-based markers (fourth generation). Proteomic technologies are rapidly evolving and are taking cancer research beyond the advances brought by genomics technologies. As opposed to genomics, the advantage of proteomics is that the identified protein/biomarker is in itself a functional endpoint and thus a true signature of a disease state. In this context, proteomics analysis has the most demanding and challenging of tasks. No amplification technology exists for proteomics. Thus, detection limits for the measurement of proteins should span the up to eight log orders of magnitude currently known to exist between the most and the least expressed proteins. In addition, proteins exhibit a wide range of biochemical properties dependent on the three-dimensional structure of the folded peptides, their biochemical stability at different pH, and a series of post-translational modifications and biochemical protein–protein interactions. Additionally, proteins may be expressed as various truncated or proteolytically modified forms. Finally, proteins, unlike RNA or DNA, do not display distinct high-affinity/high-specificity binding partners, and in most instances specific capture reagents must be developed for individual protein identification and quantitation.

Which of the currently available strategies to provide useful biomarkers and surrogates will be most useful in the clinic is an area impossible to discern at present. Below we review our assessment of the promise and problems with current strategies and try to provide a road map for investigation in the near future. It is hoped that we would be able to move beyond simple measures of tumor diameters by RECIST criteria to defining blood markers useful for assessing response to therapies at times when disease is not otherwise measurable but perhaps most susceptible to novel biologic therapies.

GENOMIC ANALYSIS OF CANCER

There are two basic approaches to the genomic analysis of cancer:

1. The analysis of genetic or epigenetic mutations that primarily drive oncogenesis. These studies are performed through an analysis of genomic DNA.
2. The analysis of functional signatures characteristic of cancer throughout its progression that could inform us about different subclinical taxonomies, possibly characterized by different natural history or responsiveness to treatment. In addition, functional genomic studies performed at salient time points may inform us about the dynamic, ongoing relationship between a cancer and the host organism and their reactions to a biologic therapy. These studies are performed by assessing gene expression profiles at the transcript or protein level in relevant specimens to provide a global phenotype of the cancer (and, in some cases, of its host).

State of the Art

With the implementation of array-based technology, high-throughput techniques have extended their boundaries to include genomic, epigenetic, transcriptomic, transcriptional regulation and proteomic analyses. This multilevel combinatorial analysis and integration of the resulting biologic information provide an unprecedented opportunity for critical insights into cancer biology and for identification of therapeutic targets. Thus, in the Workshop, we wished to emphasize the importance of this global approach (INTEGROMICS[1]) over the specific value of individual technologies. As a corollary, it may be suggested that, independent of the technology implemented, collection of samples relevant to a particular disease and its treatment should be included as a common denominator during the design of future clinical studies. These methods (with the exception of proteomics) require the same source material (DNA and/or RNA).

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CGH Arrays

Gene amplification is one of the basic mechanisms leading to gene overexpression, which is often associated with oncogenesis. Array-based comparative genomic hybridization (CGH) uses BACs (bacterial artificial chromosomes),[1a,2] cDNA clones, or oligonucleotides to hybridize according to their mapped position in a given chromosome,[3] thereby quantitating gene copy number in a test sample, as compared with a reference (normal) sample.[2,4,5] The data generated from CGH arrays are reported as fluorescence intensity ratios, which provide a cytogenetic representation of DNA copy number variations. A balanced ratio between test and reference sample suggests absence of amplification or deletion, whereas an increased or decreased ratio indicates gene amplification or deletion, respectively. This approach could be applied to the complete genome to identify known and unknown genetic alterations characteristic of distinct subsets of cancers and to monitor genomic variation through the natural history of a cancer. Identified unstable loci responsible for oncogenesis, tumor invasiveness, or metastasis can be associated with therapeutic outcomes or used for selection of molecular targets for a tailored biologic therapy. For instance, using array CGH, gene amplification studies have been carried out on primary cutaneous B-cell lymphomas, as well as on head and neck, esophageal, cutaneous squamous cell, gastric, and other carcinomas. The results not only confirmed previous reports that suggested the association of particular gene or chromosomal region amplifications or deletions with specific types of cancers, but also helped in the identification and mapping of novel genes with potential oncogenic properties. cDNA microarrays applied in conjunction with array-based CGH can then identify the relationship between genetic and transcriptional changes.

Methylation-Detection Array

Besides genetic alterations, epigenetic changes that lead to altered chromatin structure and regulation of transcription can play a causal role in tumorigenesis. DNA methylation is one the major mechanisms of epigenetic control of gene expression in cancer. Global hypomethylation of CpG dinucleotides may promote genomic instability or chromosomal rearrangements, and aberrant hypermethylation of CpG islands (CGI) in promoter regions leads to downstream gene silencing. The silencing of tumor suppressor genes mediated by hypermethylation may be relevant to oncogenesis in a manner akin to gene deletions and mutations. CGI array and differential methylation hybridization (DMH) exploit array-based technology for genome-wide screening of hypermethylated CGI in tumor cells.[6,7] These methods combine conventional methylation detection methods with array-based high-content screening. They make it possible to analyze in high throughput the prevalence of aberrant DNA methylation and may lead to the identification of biomarkers useful in the identification of populations or individuals at risk for cancer. In addition, therapeutic targeting with demethylating agents may restore growth control and may perhaps be useful as a modality for the treatment of cancer. The application of DMH and CGI array technology to the identification of the epigenetic changes associated with cancer has provided the opportunity to identify novel tumor suppressor genes. For instance, using this approach, more than 276 methylated CGI loci were discovered in a breast cancer cell line.[7] It has also been observed that SFRP1 hypermethylation can occur in normal tissues obtained from patients with colorectal cancer, suggesting that these early epigenetic aberrations not yet associated with clear phenotypic changes could be exploited as markers for the early detection of cancer and possibly for therapeutic correction.[8]

SNP Arrays

Genetic polymorphisms are often responsible for the heterogeneity of diseases and for differences in their susceptibility to treatment. For example, polymorphisms in genes such as those that code for molecules with intrinsic immune regulatory function (eg, human leukocyte antigens [HLA], cytokines, Fcγ-receptors, and killer cell immunoglobulin-like receptors) may have a significant impact on the dynamic balance between cancer progression and immune response. Other polymorphisms of genes related to cell cycle control, signaling, and transcriptional activation are only beginning to be identified; their roles in cancer progression remain to be explored. Single nucleotide polymorphisms (SNPs), which account for the majority of polymorphic sites, occur with a prevalence of 1.42 million across the human genome.[9] This dimension is becoming particularly relevant in immune biology because of the recognized polymorphisms of several genes related to adaptive and innate immune responses. Detailed discussion of high-throughput SNP identification, its impact, and its application to the biologic therapy of cancer is included in the section of this document on immune polymorphisms.

DNA–Protein Interaction Arrays

Transcriptional regulation is a key mechanism that controls homeostasis through coordination of the gene network. Gene expression levels are tightly controlled by transcriptional activators that bind to gene promoter sequences and recruit chromatin-modifying complexes and the transcriptional apparatus to initiate transcription. Global intergenic DNA fragment arrays have been developed for genome-wide location and function of DNA binding proteins.[10]

Alternative mRNA Splicing Arrays

Another interesting "specialized" array is designed to differentiate alternative mRNA splicing of the same gene or genes characterized by high homology. Yeakley et al[11] observed in a set of cancer cell lines that alternative splicing was a common feature that might be responsible for autocrine loops promoting development of the malignancies. This technology might deepen the understanding of genetic data that cannot be simply segregated by gene-specific arrays.

Gene Expression Profiling Arrays

The downstream effects of genetic and/or epigenetic variation are alterations in the level of expression of the corresponding genes and their function. Because of the intricacies of the gene regulation network, alterations in the

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expression of a single gene could lead to a cascade of downstream changes in transcription, activation, or suppression due to positive or negative feedback regulatory mechanisms. Because of this complexity, the gene expression microarray emerged as an important technology for studying complex cancer phenotypes, since it can provide unsupervised information about the parallel behavior of many genes within the human genome simultaneously in response to a given biologic situation. Thus, complex interactions between cancer cells and surrounding cells can be deduced in natural conditions or in experimental settings through the footprint of their transcriptional profiles. Dynamic portraits of the tumor microenvironment in relation to therapy can be studied[12–16] with appropriate protocol designs that include sample collection and preparation, data extraction, annotation, and integration.

Most studies to date of global transcript expression have been done with one of two types of probes. Cloned cDNA fragments (600–2,000 bp) arrays have the advantage of sensitivity, flexibly of array design, and cost effectiveness and, therefore, remain the most common custom-made platform. Synthetic oligonucleotide arrays can be divided into short oligo (25 nucleotides) arrays and long oligo (50–70 nucleotides) arrays. Tailored arrays targeted to the study of specific pathways, disease types, and treatment modalities are becoming popular.

Proteomics

Proteins, as gene end products, are the most often considered biologic effectors. Although gene expression levels often parallel the corresponding protein expression level, discrepancies are common. Complementary to functional genomics, proteomic analysis, including protein and tissue arrays, will be addressed in their respective sessions.

Application of Functional Genomics in the Clinical Setting

There are good examples of the use of clinical biopsies containing high-quality RNA and DNA for genomic and functional genomic studies of cancer and response to treatment (including immunotherapy). Most of these studies, however, have not stemmed from a prospective collection of samples, and therefore the genetic, epigenetic, and transcriptional analyses were limited to single time points, often not optimally related to the time course of a given treatment and its effects. In the future, informative data for the interpretation of biologic interactions between host, cancer, and treatment and for the identification of biomarkers will be most likely if a dynamic approach is considered in the collection of materials. Monitoring of cancer therapy will be most informative if the rationale for individual therapies is used to select critical time points for sample collection. The early application of this dynamic and multilevel approach in cancer detection should be emphasized. The identification of biomarkers will depend on this strategy and will allow that application of therapeutic modalities and their monitoring before the balance between tumor growth and the host resistance to it shifts irreversibly in favor of tumor progression.

Recommendations for Clinical Application

Genome and transcriptome analysis has been applied to a wide range of materials, from laser microdissected cells to fine-needle aspirates and to bulk tumor obtained from excisional biopsies. In clinical settings, the amount of available material is often limited, particularly when serial samples are obtained during the course of therapy. With the purpose of monitoring biologic interactions between tumor and host during immunotherapy, neither cultured cell lines nor one-time excisional biopsies could provide a dynamic view. Small biopsies such as fine-needle aspirates (FNA) from accessible lesions can be used for dynamic monitoring and prognostication. Using this approach, prospective collections of clinical samples ranging from systemic (peripheral blood) to locoregional (draining lymph nodes) to peripheral (tumor microenvironment) can be obtained without disrupting the natural history of the cancer. Collected samples should be promptly snap-frozen in the presence of RNA protection reagents such as *RNA later* or directly preserved in RNA isolation reagent at −80°C or liquid nitrogen to stop RNA degradation. We recommend that the collection of the FNA sample should be performed directly in cold physiologic solutions. This precaution minimizes RNA degradation and metabolism and allows a snapshot of the transcriptional profile in vivo. DNA and RNA amplification methods that can preserve the proportionality of individual gene expression have been developed and validated during the past few years and are well suited for this purpose. Linear RNA amplification[17–22] allows the use of samples that contain as little as nanograms of total RNA for global transcript analysis without losing fidelity in relation to the source material. Using global transcriptome analysis, biomarkers or gene expression signatures have been developed for classification of cancers[23–29] and for prediction of metastatic potential,[30] prognosis,[31] responsiveness to treatment,[14,32,33] survival,[34] chemoresponsiveness,[35–37] and treatment-induced gene activation or suppression.[38] Thus, it is likely that expression profiling will play a dominant role in suggesting successful treatment modalities based on biologic mechanisms.

Data Analysis and Annotation

The meaning of microarray data is highly dependent on the experimental design, array platform, sample preparation, method of data analysis, and annotation for biologic interpretation. Data analysis begins with background (local or global) subtraction and data normalization (by global ratio, total intensity, linear regression, curvilinear analysis, or internal controls). Subsequent data analyses include class discovery, class comparison, and class prediction. For class discovery, the most commonly used analytic methods include hierarchical clustering, K-mean clustering, self-organizing maps (SOM), and principal component analysis (PCA). Class comparison employs preexisting knowledge to examine differences among experimental groups. Methods used for this purpose include parametric tests such as the Student *t* test for two categories and analysis of variance (ANOVA) for more than two categories. Stepwise multivariate permutation tests[39,40] have been proposed to control the family-wise error

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rate and control the number or proportion of false discoveries. Global permutation tests can be used to test whether there is an overall difference in average expression profile between classes without specifically identifying genes responsible for those differences. Class prediction aims to develop a multivariate class predictor for predicting the phenotype of a new sample. Methods applied in microarray class prediction include Fisher linear discriminant analysis,[39] diagonal linear discriminant analysis and its variants (weighted voting methods[41] and compound covariate prediction[42]), nearest neighbor classification,[43] regression trees,[44] neural networks,[45] and support vector machines.[46] The accuracy of a class predictor can be estimated by using the training set predictor to foretell the phenotype in the test set. With small sample size, leave-one-out cross-validation can be used as an alternative.

Websites Available

Compatible array analysis software including Significance Analysis of Microarrays (SAM)[47] (http://www-stat.stanford.edu/~tibs/SAM/), BioConductor (http://www.bioconductor.org/), and BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) are publicly accessible and cover most of the statistics, bioinformatics, and visualization tools. The biologic interpretation of array data is the major gap between statistics and bioinformatics. GoMiner[48] (http://discover.nci.nih.gov/gominer) is one of the programs that attempts to fill the intellectual gap between statistical identification of significant data and their biologic interpretation. This package includes a freely available computer source that fully incorporates the hierarchical structure defined by the Gene Ontology (GO) Consortium to automate the functional categorization of the gene lists generated by individual studies. This software links to LocusLink, PubMed, MedMiner, GeneCards, the NCBI's Structure Database, BioCarta, and KEGG pathway maps. MatchMiner (http://discover.nci.nih.gov/matchminer/html/index.jsp) enables the user to translate between disparate IDs for the same gene. It uses data from the UCSC, LocusLink, Unigene, and OMIM data sources to determine how different IDs relate. Supported ID types include gene symbols and names, IMAGE and FISH clones, GenBank accession numbers, and UniGene cluster IDs. Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://apps1.niaid.nih.gov/david/upload.jsp), developed by NIH, annotates genes with different IDs and links with GO charts, KEGG charts, and domain chart analysis. GenePublisher[49] (http://www.cbs.dtu.dk/services/GenePublisher/) has been implemented with a web interface for automatic array normalization, statistical analysis, data visualization, and gene annotation. Final output results are reported after annotation via LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/) databases and the GO (http://www.ebi.ac.uk/GOA/). Interesting genes identified are also linked to databases such as Protfun (http://www.cbs.dtu.dk/services/ProtFun/) for predicting gene function, KEGG (http://www.genome.ad.jp/kegg/) for metabolic pathway matching, and TRANSPATH (http://www.biobase.de/pages/products/transpath.html) for signal transduction pathway matching.[50]

Standardization and Validation of Array Analysis

As different array platforms, experimental designs, material preparations, and analysis tools are employed more widely, data comparison becomes a daunting and often frustrating task. Not only the comparison but also the validation of genes identified as biologic markers is often viewed skeptically. The demand for standardization of microarray data extraction and interpretation is rapidly increasing as large databases accumulate. Minimum Information About a Microarray Experiment (MIAME)[51] is a first approach to standardization of array data and metadata presentation, formulated by the Microarray Gene Expression Data group (MGED). It has been proposed for microarray data collection, archiving, and public access.

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DETECTION OF MOLECULAR MARKERS IN PERIPHERAL BLOOD BY RT-PCR

State of the Art

Reverse transcriptase–polymerase chain reaction (RT-PCR) has been used to amplify cancer-specific gene expression as a means of detecting occult tumor cells in a variety of tissues, including lymph nodes, bone marrow, and peripheral blood. The literature suggests that detecting a molecular footprint of occult tumor cells in lymph nodes and/or bone marrow may be informative in several cancers, such as melanoma[1,2] and esophageal[3] and breast[4] cancers. The detection of circulating tumor cells (CTCs) in the peripheral blood is considered to be more desirable and has been reported for many cancers, including prostate, breast, melanoma, gastrointestinal, acute myeloid leukemia, and neuroblastoma.[5–13] These studies have mostly demonstrated a positive correlation between CTC detection and advanced cancer stage or a higher incidence of disease relapse.

The detection of CTCs by RT-PCR has been successful, in part, on the identification of marker genes for each cancer (Table 1). For many types of cancers, a single molecular marker has been used for the detection of CTCs. For example, prostate-specific antigen (PSA) has been reported to be a consistently reliable, single marker for prostate cancer prognosis.[14,15] The clinical value of recently identified marker genes may still be undetermined, such as Lunx gene expression, where detection in the peripheral blood has been correlated with non-small cell lung carcinoma (NSCLC) but has not yet been correlated with clinical stage or relapse potential.[16] In contrast, the use of a single marker for melanoma, such as tyrosinase, has been determined by some to be inconsistently correlated with stage or relapse incidence.[17] For melanoma, the use of multiple markers, such as tyrosinase, p97, and MelanA/MART-1, has been developed for more accurate detection of CTCs in peripheral blood.[5,18,19] This lack of a definitive, single marker gene for some cancer types, such as melanoma, may be a function of either genetic drift or selective pressure by the immune system against immunogenic proteins produced by cancer cells.[18]

In addition to CTC detection, an emerging use of RT-PCR has been the characterization of immune responses to malignancy. The detection of immune cell markers by RT-PCR has enabled the detection and quantification of immune cells in diseased tissues such as in tumors,[20] sentinel lymph nodes,[21] and allograft biopsies.[22,23] These studies report that the detection of dendritic cell activation markers, T-cell markers, or cytokine expression in biopsies enables the characterization of the immune response for its potential impact on clinical

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TABLE 1. Molecular Marker Candidates for Circulating Tumor Cells in Peripheral Blood

Cancer	Molecular Marker (mRNA)	Reference
Melanoma	Tyrosinase, p97, MUC18, and MAGE-3	(5)
	MAGE-A12	(54)
	Tyrosinase and Melan-A	(18, 55)
	Tyrosinase, Melan-A and universal melanoma antigen gene-A (uMAG-A)	(19)
Breast	Parathyroid hormone-related protein	(6)
	Human mammaglobin	(56)
	CK-19 and maspin	(57)
	MUC1	(8)
	Epithelial glycoprotein-2 (EGP-2) and CK19	(58)
	Sialyltransferases	(59)
	CK-20	(60)
	mam, PIP, CK-19, mamB, MUC1, CEA	(4)
Prostate	Human cachexia-associated protein (HCAP)	(61)
	Prostate-specific Ets factor (PSE)	(50)
	Prostate-specific antigen (PSA)	(9, 14, 15, 62)
Gastrointestinal	CEA, CK19, and CK20	(10)
Neuroblastoma	Tyrosine hydroxylase	(11)
Non-small cell lung	Lunx	(16)
carcinoma (NSCLC)	EGFR	(63)
AML	Multidrug resistence protein 1 (MDR1)	(12)
APL	CD34	(64)
Renal cell carcinoma	Cadherin-6	(65)
Colorectal	CEA	(37, 66)
	CK-20	(67)
Cervical	Epidermal growth factor receptor (EGFR)	(68)
Thyroid	Thyroglobulin	(69)
	Human kallikrein 2 (hK2)	(70)
	Calcitonin	(52)
	Thyroid peroxidase, thyroglobulin, RET/PTC1	(71)
Esophageal	CEA	(72)
	Squamous cell carcinoma (SCC) antigen	(73)
Pancreatic	EGFR	(63)
Head and neck	E48, Ly-6	(74)
Gastric	hTERT	(75)
Hepatocellular carcinoma	MAGE-1, MAGE-3	(76)

The molecular markers itemized in this table have been used to detect CTCs in human peripheral blood and were reported to correlate with clinical stage or exhibit specificity for the indicated cancer as compared with normal individuals or individuals with unrelated cancer.

outcome. RT-PCR analysis of immune cell responses in the peripheral blood, as compared to analysis of tissue biopsies, has primarily been focused on the detection of functional markers. These functional markers include the detection or quantification of cytokine expression,[24–26] HLA polymorphisms,[27] activation marker expression on T cells (eg, perforin, Ox40)[21,28,29] and TCR usage.[30]

Establishing cytokine expression profiles in the peripheral blood by real-time RT-PCR to characterize the immune response has increased in recent years. The quantification of interferon (IFN)-gamma, interleukin (IL)-2, IL-4, IL-5, IL-6, and IL-10 mRNA from peripheral blood mononuclear cells (PBMCs) enables the assessment of Th1 or Th2 bias[31] and activation status of effector cell populations[32] in cancer patients. Cytokine mRNA profiles established by real-time RT-PCR are reported to be quantitatively and qualitatively similar to profiles established by enzyme-linked immunosorbance assay (ELISA).[20] Real-time RT-PCR detection of cytokines has also been applied to quantify antigen-specific cellular immune responses in the peripheral blood after active-specific immunotherapy. Blood or PBMCs isolated from immunized patients are restimulated with the antigen and IFN-γ mRNA is subsequently quantified by RT-PCR.[33–35]

These applications exploit the primary strengths attributable to RT-PCR for the analysis of blood samples—that is, its high-level sensitivity and ability to evaluate the expression of multiple genes from a small sample size. The limit of detection (LOD) for tumor marker genes has been reported to be one CTC in a milliliter of whole blood or 1 to 10 tumor cells in 10^6 or 10^7 lymphocytes or PBMCs.[7,36–38] This level of sensitivity gives RT-PCR a competitive advantage for the detection of CTCs over methodologies that rely on immunodetection methods such as imaging or flow cytometry techniques. An important caveat is that the actual LOD may be lower in patient

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samples. The estimation of assay sensitivity necessarily uses tumor cell lines that may have high marker mRNA steady-state levels, while CTCs in cancer patients may have only a few copies of the same marker mRNA. The amplification of multiple markers from a single RNA sample by multiplex amplification reactions[39] and the simultaneous analysis of multiple samples[40] are strong attributes of real-time RT-PCR. These attributes result in the ability to amplify multiple tumor marker genes or cytokines to maximize the information content contained in a single blood sample in a timeframe consistent with clinical settings.[40]

Clinical Application

As a molecular diagnostic application, RT-PCR has the demonstrated potential to improve evaluation of peripheral blood samples for occult tumor cells and immunologic function in cancer patients. However, before these applications can be used as an approved clinical endpoint, rigorous performance validation is required. The categories for performance validation include standardization of sample processing and RT-PCR assay conditions; establishment of assay controls; and the design and implementation of prospective clinical studies to validate RT-PCR quantification of markers expressed by CTC or immune response cells.

Sample Processing

Optimal RT-PCR performance is dependent upon sample processing, and there are multiple aspects to sample processing that influence assay performance. The most important aspect of sample processing determined by an EORTC Melanoma Cooperative Group discussion was the rapid preservation of sample RNA.[41] Isolation of RNA from whole blood was preferable to density gradient purification prior to RNA isolation and provides the easiest process for validation since there are few sample processing steps and minimal RNA degradation.[41,42] The inclusion of an RNA preservative in the blood collection tube improves assay sensitivity and facilitates standardization and quality control.[43,44]

Detection of Circulating Tumor Cells

Detection of CTCs in the PBMC fraction after Ficoll gradient centrifugation provides an alternative to whole blood processing as it partially enriches for CTCs.[5] One of the drawbacks of Ficoll purification of PBMCs is the variability reported between clinical laboratories. This variability may be reduced through the use of collection tubes designed for one-step PBMC processing.[42] Additional methodologies for the further enrichment of CTCs from the peripheral blood have been reported to enhance the sensitivity of RT-PCR analysis. These methodologies include porous density gradient centrifugation,[8] positive or negative selection of CTCs using magnetic microbead technology,[7,45] and isolation of CTCs by size.[46] The inclusion of additional enrichment steps may adversely affect interassay and interlaboratory variability. A further disadvantage of using an enrichment step might be a loss of at least a proportion of the total number of tumor cells present in the original sample.

RT-PCR

Rigorous validation of RT-PCR reagents and assay conditions is imperative for consistent performance between laboratories. With the availability of multiple marker genes for each cancer type, validation efforts must include the identification of a single gene or set of genes as the standard marker for each cancer. While this decision might be readily agreed upon for certain marker genes such as PSA for prostate cancer,[14,15] it is likely to be more difficult for cancers where multiple markers exist, as is the case for melanoma. In addition, validation and standardization of each process of RT-PCR must be performed. These include validation of reverse transcriptase primers, reverse transcription assay conditions, and real-time PCR primers, probes, and amplification reaction conditions.

The incorporation of real-time PCR for molecular diagnostics has a number of advantages over conventional PCR. Real-time PCR is more amenable to validation and standardization since it has fewer hands-on steps than conventional PCR, resulting in more consistent intra-assay and interassay results.[12,47] This attribute suggests the possibility for automation of many of the steps required for RT-PCR analysis. The quantitative capabilities of real-time PCR provide additional benefits in the analysis of peripheral blood RNA samples. The quantification of marker gene expression by CTCs may provide an added level of prognostic power beyond the detection of a CTC in a patient's blood. However, whether marker gene expression levels are correlated with clinical outcome such as relapse potential needs further investigation. The quantification of cytokine expression is, however, is more pressing for accurate evaluation of the immune response, since mRNA levels of many cytokines are endogenously expressed in leukocytes isolated from the peripheral blood of normal donors.

Controls

RNA integrity is assessed by spectrophotometry and amplification of an internal standard, such as a housekeeping gene. It is not necessary to restrict selection of housekeeping genes to those having low copy number with real-time PCR, because high copy housekeeping genes can be monitored using low cycle number threshold. Candidate low copy and high copy genes include porphobilinogen deaminase[48] and GAPDH, respectively. Alternatively, artificial transcripts and synthetic oligonucleotide mimics that have the same primers as the target mRNA can also serve as internal standards.[48,49]

Clinical Validation

The establishment of real-time RT-PCR as an endpoint for the detection of CTCs and surrogate marker of disease state and prognosis must be validated in multicenter clinical trials. Clinical trial objectives for RT-PCR validation include (1) sensitivity and specificity of RT-PCR detection of marker mRNA, (2) accuracy of CTCs for staging and prognosis, (3) monitoring changes in CTC levels in response to therapeutic treatment, (4) correspondence of CTCs with overall survival after therapeutic treatment, and (5) comparison of CTCs in peripheral blood with marker detection in draining lymph nodes and bone marrow. The inclusion of serial analysis of

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clinical samples in the trial design would provide variability in signal-to-noise ratio over time. Comparison of RT-PCR performance between laboratories will provide the necessary documentation specific to assay validation. A clinical strategy for the establishment of immunologic marker quantification as an immunologic endpoint would be similar to that described for CTC detection. This strategy includes (1) monitoring changes in immunologic marker gene expression profiles over the course of disease and (2) correspondence of immunologic marker gene expression with overall survival in response to therapy. Rigorous SOPs must be established for each process in the analysis, and RT-PCR analysis of clinical samples should be run at multiple laboratories to assess interlaboratory variability. Uniform internal standards and methodology for RNA isolation will facilitate reproducible and valid results. Finally, standardized interpretation of results is required to establish uniform agreement on definitions of positive and negative results.

Opportunities for Advancement

RT-PCR analysis of blood for CTCs and immunologic responses has benefited from the rapid advances in molecular technologies. Serial analysis of gene expression (SAGE) and real-time PCR have identified novel marker genes overexpressed by CTCs, such as the prostate-specific Ets (PSE) factor.[50] Microarray analyses of tumors or PBMCs in responding and nonresponding cancer patients after immunotherapy are providing novel marker genes that may serve as diagnostic marker genes or as therapeutic targets. Finally, these molecular technologies may also provide information on the biology of CTCs to better predict chemosensitivity[51] and metastatic potential.[52,53]

New high-throughput technologies will facilitate assay standardization. For example, immune profiling cards may enable investigators to quantitatively evaluate the expression of larger numbers of genes to establish a more accurate portrait of the immune response and its impact on tumor progression. These cards are self-contained chambers that can contain primers and probes for quantification of different numbers of targets, samples, or replicates in a single assay. New automation technologies are being developed that will reduce the amount of sample manipulation, which reduces interoperator variability and produces more consistent analyses between individual clinical laboratories.

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SERUM, PLASMA, AND TUMOR PROTEOMICS

State of the Art

In the context of cancer research, proteomics encompasses a broad range of biologic variables that include the characterization of proteins or peptides derived from the host, the tumor microenvironment, and the peripheral circulation, which may constitute metabolic fragments resulting from the metabolism, enzymatic degradation, or protein–protein interactions of cancer cells.[1,2] Serum, plasma, saliva, urine, cerebrospinal fluid (CSF), skin blister fluids, feces, peripheral blood mononuclear cells, tumor tissue (excisional biopsies or fine-needle aspirates), and lavage fluids (bronchoalveolar lavage, nipple aspirate fluids, gynecocervical lavage, and ascites) represent the major sources of biologic material accessible for cancer biomarker discovery. Table 2 summarizes the state of the art of proteomic strategies aimed at characterizing the modified or unmodified proteins involved in cancer progression. Methodologies used for biomarker discovery include two-dimensional gel electrophoresis (2D-GE), surface-enhanced laser desorption time-of-flight mass spectrometry (SELDI-TOF-MS),[3–5] liquid chromatography/mass spectrometry (LC-MS/MS), isotope coded affinity tag (ICAT),[6] multidimensional protein identification technology (Mud PIT, which couples 2D-LC to MS/MS),[7–9] laser capture microdissection (LCM) tissue arrays, and quantitative protein arrays.[10–12]

A summary of the most recent achievements in cancer biomarker detection and the associated proteomic technology, including removal of high abundance proteins,[13,14] is shown in Table 3. This manuscript and Table 3 do not address biomarkers already in use in the clinical setting for cancer screening, diagnosis, and monitoring, since the purpose of this workshop is to discuss advances and the future of biomarker discovery. We refer to a summary by Sturgeon that provides condensed and relevant information on the topic.[15]

Table 4 and Figure 1 summarize the most common problems/obstacles encountered in protein identification and analysis when one of the most commonly accessible biological materials (serum/plasma) is studied (Table 4); in addition, possible solutions are discussed across different types of samples (Fig. 1). Refinements will be necessary, such as the elimination of high abundance proteins,[13,14] enrichment of low-level protein families, and the use of high-sensitivity and -specificity technologies such as HPLC and mass spectrometry for the detection of rare plasma proteins that could be used as biomarkers.[7] Meanwhile, none of the individual technologies is problem-free and capable of reliably identifying relevant proteins in a wide range of experimental situations. Possibly a combination of different methodologies will be more successful in biomarker discovery.

What Should Be Applied Now in the Context of Clinical Trials or Conventional Practice?

Assuming that a new cancer biomarker (see Table 3) has been identified either as an individual protein or as a pattern profile,[16–50] the proteomic technology to be applied for its screening will depend on the careful collection of appropriate samples and highly validated and characterized methods of sample analysis. The resources available for these studies may be beyond the reach of conventional clinical practice. Additionally, the prohibitive cost of several proteomic technologies may limit their application to a few institutes in which core facilities can be organized.

Conventional Clinical Practice

Screening of established biomarkers[15] by classical ELISA is probably the only proteomic methodology currently adopted in clinical practice. Signature of disease state according to multiprotein platforms is certainly an option in the near future. However, standardization and validation will need to be performed in large scale in experimental settings before there could be wide application to clinical situations (see below). Screening for new biomarkers may not be used for diagnostic purposes until full protein characterization and highly specific antibodies are developed that could make the marker more easily detectable using standard technologies.

Clinical Trials

Screening for novel biomarkers realistically can be afforded by large research institutions in the setting of experimental trials. State-of-the-art proteomic technology can be applied in these settings because qualified experts in the field of protein biochemistry, high-throughput proteomics, and bioinformatics can simultaneously contribute to the preparation, testing, analysis, and interpretation of data derived from serum, plasma, and tumor tissue specimens.

Upon identification of putative biomarkers, three major issues need to be resolved before a novel biomarker can be introduced in clinical trials with the purpose of early detection, diagnosis, monitoring of therapy, and prevention or risk assessment of cancer: the feasibility (sensitivity, specificity, and selectivity for measuring natural protein and its post-translationally modified forms); reproducibility (using established controls, day-to-day variability, precision, matrix

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TABLE 2. State of the Art Technologies in Proteomic Diagnostics: Comparison of Proteomics Technologies and Their Contribution to Biomarker Discovery.

ELISA	2D GE PAGE	Isotope Coded Affinity Tag ICAT*	Mud PIT†*
Chemiluminescence-based Fluorescence-based	2D GE PAGE DIGE Serological proteome analysis (SERPA) 2D-GE + serum immunoblotting	ICAT/LC-ECI-MS/MS ICAT/LCI-MS/MS/MALDI	2D LC-MS/MS*
Sensitivity			
Highest	Low particularly for less abundant proteins, sensitivity limited by detection method LCM can improve specificity via enrichment of selected cell population Difficult to resolve hydrophobic proteins	High	High
Direct Identification of Makers			
N/A	YES	YES	YES
Use			
Detection of single well characterized specific analyte in plasma/serum, tissue, gold standard of clinical assays	Identification and discovery of biomarkers not a direct means for early detection in itself	Quantification of relative abundance of proteins from two different cell states	Detection and ID of potential biomarkers
Throughput			
Moderate	Low	Moderate/low	Very low
Advantages/Drawbacks			
1) Very robust, well established use in clinical assays 2) Requires well characterized antibody for detection 3) Requires extensive validation not amenable to direct discovery 4) Calibration (standard) dependent 5) FDA regulated for clinical diagnostics	1) Requires a large amt of protein as starting material 2) All ID require validation and testing before clinical use 3) Reproducible and more quantitative combined with fluorescent dyes 4) not amenable for high throughput or automation 5) Limited resolution, multiple proteins may be positioned at the same location on the gel.	1) Robust sensitive and automated 2) suffers from the demand for continuous on the fly selection of precursor ions for sequencing 3) coupling with MALDI promises to overcome this limitations and increase efficiency of proteomic comparison of biological cell states {Flory et al, 2002} 4) Still not highly quantitative and difficult to measure sub-pg/ml concentrations	1) Significant higher sensitivity than 2D-PAGE 2) Much larger cover age of the proteome for biomarker discovery 3) Still limited for low abundance proteins and low molecular weight fractions

ELISA	Proteomic Pattern Diagnostics*	Protein Microarrays	Enhanced Binding Surfaces*	Inductively Coupled Plasma MS Immuno-assay ICP-MS
Chemilumines cence-based Fluorescence-based	MALDI SELDI-TOF-MS SELDI-QqTOF	Antibody arrays 1) Chemiluminescence Multi Elisa platforms, 2) Glass Fluorescence based (Cy3-Cy5) 3) Tissue arrays	Sensor chips Surface Plasmon Resonance (SPR) SELDI antibody treated chip	Antibody array with element tagged antibodies ICP-MS
Sensitivity				
Highest	Medium-to-high sensitivity. SELDI-TOF-MS and MALDI are both capable of analyzing a wide mw range of proteins with generally diminishing signal at higher masses. Compact MS systems typically yield a sensitivity benefit as a trade-off for resolution; shorter drift tubes allow more ions to reliably reach the detector. SELDI-QqTOF technology with distinct resolution advantages has been shown to have comparable sensitivity in low mass range, but has an effective cut-off in analysis at fairly low masses.	Medium to highest (depending on detection system)	High	High?
Direct Identification of Markers				
N/A	Molecular mass of intact proteins or peptides yields a very tentative ID while the principle of SELDI pattern diagnostics conveniently defines a path to rapid marker enrichment for the purposes of ID (Nakamure et al, 2002). The SELDI-QqTOF platform provides a more direct route to protein ID by	Possible when coupled to MS technologies; or probable, if antibodies have been highly defined by epitope mapping and neutralization	YES	YES with secondary MS

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TABLE 2. (*continued*) State of the Art Technology in Proteomic Diagnostics: Comparison of Proteomics Technology and Their Contribution to Biomarker Discovery.

ELISA	Proteomic Pattern Diagnostics*	Protein Microarrays	Enhanced Binding Surfaces	Inductively Coupled Plasma MS Immuno-assay ICP-MS
	enabling on-chip tandem MS for peptide sequencing. Some complete protocols for on-chip marker purification, proteolysis and MS/MS ID have been shown (Caputo et al, 2003). Rapid, on-chip immunoaffinity identification protocols for SELDI when antibodies exist to tentatively identified markers.			
		Use		
Detection of single well characterized specific analyte in plasma/serum, tissue, gold standard of clinical assays	Diagnostic pattern analysis in body fluids (serum, urine, CSF, feces, etc.) and tissue (with or without LCM). Potential biomarker identification. SELDI protein interaction mapping for functional studies as well as biomarker assays (with specific bait protein coupled to chip).	Multiparametric analysis of many analytes simultaneously	Protein-protein interaction analysis Identification of disease markers in clinical samples Quantitative measurement of binding interaction and specificity between molecules, ligand fishing(??)	Multiparametric analysis (limited protein number to date) with MS detection
		Throughput		
Moderate	High	High	High	?
Advantages/Drawbacks				
1) Very robust, well established use in clinical assays 2) Requires well characterized antibody for detection 3) Requires extensive validation not amenable to direct discovery 4) Calibration (standard) dependent 5) FDA regulated for clinical diagnostics	SELDI 1) Protein ID not necessary for biomarker pattern analysis, but patterns narrow down the relevant proteins for ID studies while the SELDI process is useful to defining a rapid isolation and ID protocol 2) Reproducibility and quantitative performance better than MALDI; debate exists over current reproducibility achieved from site-to-site 3) Rapid analysis and parallel processing of large sample populations possible 4) Revolutionary tool; as little as 1–2 uL amount of material required; slightly more (20 µL) with prefractionation procedures 5) As with other technologies, SELDI works synergistically with upfront fractionation of serum and other complex samples–fractionation increases the number of proteins detected at a cost of time and amount of sample; downstream purification methods necessary to obtain absolute protein identification MALDI 1) Commonly available equipment can be employed to combine off-line LC with MALDI for proteomic pattern generation 2) Matrix crystallization procedure is a large source of irreproducibility and can be matrix and sample dependent 3) High mw proteins often not directly detectable requiring global digestion and shot-gun MS/MS approaches	1) Format is flexible Can be used to assay for multiple analytes in a single specimen or a single analyte in a number of specimens 2) Requires prior knowledge of analyte being measured; 3) Limited by antibody sensitivity and specificity; 4) Requires extensive crossvalidation for antibody crossreactivity 5) Require use of an amplified tag detection system 6) Requires more sample to measure low abundant proteins, needs to be measured undiluted	New technology 1) Can be used to assay for a single analyte in a number of specimens 2) SELDI chip limited by antibody sensitivity and specificity 3) Hardware limitation with the PBS II vs the QqTOF 4) Promising combined strategy of protein array chip and MS.	New technology Very early stage 1) Non-amplified, analysis of element allows for direct measurement without background or contamination 2) Acidification of sample allows archiving. 3) May require too much sample

*State of the art technologies in proteomic diagnostics. Abbreviations for Table 2, 3, 4: 2D GE, 2 Dimensional Gel Electrophoresis; DIGE, differential in gel electrophoresis; SDS–PAGE, Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis; PAGE, Polyacrylamide Gel Electrophoresis; SERPA, Serological Proteome Analysis; ICAT, Isotope Coded Affinity Tag; MudPIT, Multidimensional protein identification technology; MALDI, Matrix Assisted Laser Desorption lonization; SELDI-qTOF, Surface Enhanced Laser Desorption Ionization Quadrupole time of flight; ESI, Electrospray Ionization; ESI FTICR, Electrospray Ionization Fourier Transform Cyclotron Resonance; LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry; SRP, Sensor Chip Surface Plasmon Resonance; LCM, Laser Capture Microdissection; MUDPIT, Multidimensional protein identification technology; ICH, Immunoaffinity subtraction chromatography; IHC, Immuno histochemistry; TARP, Arrays Tissue Array Research Program; UMSA, Unified Maximum Separability Analysis.

Modified from Wulfkuhle et al, *Nature Reviews*, 2003.

effects, etc.); and standardization (calibration of instrument and standards [recombinant or natural]) of the selected technology in detecting the biomarker in question. In fact, to our knowledge, none of the state-of-the-art technologies summarized in Table 2 and used in the studies reported in Table 3 has yet fully satisfied these three criteria and can be convincingly applied in clinical trials. The workshop participants could not reach a consensus on whether it is best to collect serum or plasma for proteomic studies until information is available to recommend one. Therefore, when possible, a recommendation would be to collect both. A common reference sample to use across different studies was deemed to be a requirement for all types of mass spectometric analysis as well as multiprotein arrays. We suggested generation of a reference standard consisting of "a pool of different serum standards for each ethnic background

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TABLE 3. Achievements in Cancer Biomarker Detection

Reference	Type of Cancer/ Clinical Study	Source of Biological Material	Proteomic Technology of Choice	Biomarker/ Surrogate	Significance and Potential
Petricoin III et al, 2002	Ovarian	Crude serum	SELDI-TOF C16 Chip Bioinformatics/pattern generation; use of genetic algorithm to obtain a pattern that best segregates between training sets of spectra from sera of ovarian cancer and normal *New recent development:* High resolution mass spec ABI Hybrid Pulsar QqTOF (Q-Star) fitted with Ciphergen SELDI	Cluster pattern	Identification of finding justifies prospective population-based assessment of proteomic pattern technology as a screening tool for all stages of ovarian cancer in high risk and general population. Problems with spectra alignment machine to machine variance, and reproducibility. New adjustment to the configuration of SELDI introduced an enormous increase in resolution and increase in mass accuracy. Introduction of the Q-Star has the potential to allow for sequence analysis and identification of the ions that comprise the diagnostic information.
Kim et al, 2002	Epithelial ovarian	Preoperative plasma, tumor tissue, control normal ovarian tissue	MICROMAX RNA array. Immunohistochemistry (IHC) Laser capture microdissection (LCM) ELISA	Osteopontin	Invasive ovarian cancer tissue and borderline ovarian tumors higher levels of osteopontin than benign tumors. Osteopontin level in plasma of epithelial ovarian cancer significantly higher than controls, benign ovarian disease and other gynecological cancers. Potential useful screening marker for early asymptomatic disease.
Nishizuka et al, 2003	Ovarian, colon Multistep protocol NCI*	Ovarian and colon cell lines	Multistep protocol: 1) cDNA microarrays 2) clone verification by resequncing 3)Affimetrix oligo chips *4) Reverse protein lysate microarrays 5) Validation on candidate markers by TARP tissue microarrays*	Villin (colon cancer) Moiesin (ovarian cancer)	Potential for Differential diagnosis of colon and ovarian malignancies, discrimination of colon from ovarian carcinoma in ovarian masses, peritoneal carcinomatosis and metastasis to distant lymph nodes. Multistep Process potential to produce additional markers for cancer diagnosis, prognosis and therapy.
Kozak et al, 2003	Ovarian	Unfractionated serum	SELDI-TOF-MS; SAX2 Protein Chip Arrays	Three recursively partitioned pattern models generated comprised of a total of 13 protein peaks	Both the potential for early detection screening and differentiation of benign versus malignant disease is presented. ROC areas under the curve were in the range of .90 to .94. Contrary to other reports of SELDI and MALDI having limited utility in a relatively low mass range of detection, the 13 protein peaks uncovered in this study ranged up to 106.7 KDa.
Ye et al, 2003	Ovarian	Unfractionated Serum Ovarian cell lines	SELDI-TOF-MS IMAC-3 chip Affinity chromatography SDS-PAGE LC-MS/MS for aa sequence. Peptide synthesis and Specific antibody development PCR and western blot testing for overexpression in tumor cells	Pattern profiling + identification of protein in discriminatory patterns A serum biomarker at ~11,700 Da was identified as the α chain of haptoglobin	Elevated levels of Hp-α in ovarian cancer patients seen. As a single marker, the predictive power of Hp-α appeared lower than CA-125, but may be complementary to it in a multi-marker profile. A candidate biomarker was of 11.7 KDa was rapidly picked up in this study using the SELDI platform; ID was accomplished with a combination of affinity column enrichment, SELDI monitoring of fractions, digestion and LC-MS/MS. Protein profiling valuable tool for screening potential biomarkers but confirmation of protein identity with specific antibodies and classical immune assays is crucial for clinical application and functional studies. Proteolytic cleavage of Hp a from b detected in the presence of cancer serum only, and not in cancer cells, Hp a subunit elevation caused by specific enzymatic cleavage and abnormal protein-protein interaction in the circulation of cancer patients rather then in the tumor. Evidence for potential detection of metabolic peptide biomarkers and post translational modifications by proteomics technologies.

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TABLE 3. (*Continued*) Achievements in Cancer Biomarker Detection

Reference	Type of Cancer/ Clinical Study	Source of Biological Material	Proteomic Technology of Choice	Biomarker/ Surrogate	Significance and Potential
Rai et al, 2002	Ovarian	Plasma	SELDI-TOF-MS, H4, NP, IMAC3 Arrays, SELDI-QqTOF, Biomarker Patterns analysis	7 biomarkers: 8.6, 9.2, 19.8, 39.8, 54, 60, 79 KDa. Only the three peaks at 9.2, 54, and 79 KDa could be identified; the 79 kDa peak corresponded to transferrin, the 9.2 kDa peak corresponded to a fragment of the haptoglobin precursor protein, and the 54 kDa peak was identified as immunoglobulin heavy chain.	The combined use of bioinformatics tools and proteomic profiling provides an effective approach to screen for potential tumor markers. Comparison of plasma profiles from patients with and without known ovarian cancer uncovered a limited panel of potential biomarkers. These biomarkers, in combination with CA125, provide significant discriminatory power for the detection of ovarian cancer.
Jones et al, 2002	Ovarian	Invasive ovarian cancer and non invasive low malignant potential (LMP)	LMC 2D-PAGE Reverse phase array technology	FK506, RhoG protein dissociation inhibitor Gyoxalase I	Direct comparison of LCM generated profiles of invasive vs LMP cancer, directly generated important markers for early detection and/or therapeutic targets unique to the invasive phenotype
Li et al, 2002	Breast	Serum	SELDI-TOF IMAC3 chip Bioinformatics to achieve best pattern selection: unified maximum separability analysis (UMSA) algorithm + Bootstrap crossvalidation with introduction of random perturbations ProPeak	Three distinct pattern profiles Putative biomarkers BCI = 4.3 kDa, BC2 = 8.1 kDa, BC = 8.9 kDa	Identification of potential biomarkers that can detect breast cancer at early stages: separation between stage 0–1 and non cancer control AUC composite index for the 3 markers panel was 0.972, The best single marker (BC3) showed an AUC of 0.934.
Caputo et al, 2003	Breast	Breast cyst fluid	SELDI-TOF-MS, H4, PS10 Arrays	Pathological differences between similar proteins GCDFP-15/PIP and physiological gp17/ SABP shown.	SELDI used to investigate interaction with these proteins and CD4 and FN. It was determined that the physiological form was involved with the binding to CD4. Depending on its conformational state, GCDFP-15/gp17 could differentially bind to its various binding molecules and change its function(s) in the microenvironments where it is expressed.
Vlahou A	Breast	In press	In press	In press	In press
Yousef et al, 2003	Breast, Ovarian	Serum	Recombinant protein ELISA	Human Kallikrein 5 (hK5)	Development of first fluorimetric assay for hK5, distribution of hK5 in biological fluids and tissue extracts. Potential valuable diagnostic and prognostic marker for ovarian and other cancers
Sauter et al, 2002	Breast	Nipple Aspirate	SELDI-TOF-MS, H4, NP1, SAX2 Arrays	Five differentially expressed proteins were identified. The most sensitive and specific proteins were at 6500 and 15940 Da.	Analysis of nipple aspirate fluid proteins by SELDI may predict the presence of breast cancer.
Govorukhina et al, 2003	Squamous cervical cancer	Serum depleted of albumin and γ-globulin	LC/MS ELISA	SCCA1	SCCA1 is low abundance protein although ELISA is commercially available, this study shows that this type of proteins can be successfully detected by LC-MS following depletion of albumin and g globulin LC-MS promising technique for biomarker discovery
Leher et al, 2003	Prostatic neoplasm	Crude Serum	SELDI-TOF	Novel 3 proteins 15.2, 15.9, 17.5 kDa	15.9kDa molecule maybe used for diagnosis of PC vs benign prostatic hyperplasia (BPH). Potential for antibody based chip SELDI-TOF

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TABLE 3. (*Continued*) Achievements in Cancer Biomarker Detection

Reference	Type Cancer/ Clinical Study	Source of Biological Material	Proteomic Technology of Choice	Biomarker/ Surrogate	Significance and Potential
Hlavaty et al, 2003	Prostatic intraepithelial neoplasia (PIN)	Serum purified of lipids, IgG, human serum albumin (HSA) and fractionated by HPLC/anion exchange column	SELDI-TOF (WCX2 chip) SDS-PAGE, tryptic digest Peptide mass fingerprinting	Novel 50.8 kDa protein NMP48	Detection in PIN not in BPH or controls Assay for NMP48 maybe useful for early detection of prostate cancer
Banez et al, 2003	Prostate Cancer (CaP)	Crude serum	SELDI-TOF Combination of IMAC3-Cu and WCX2 chips Biomarker Pattern Software (BPS)	Combination of pattern profiles from two different chips	Combined effect of using 2 array types enhances the ability of using protein profile patterns for CaP detection
Cazares LH et al, 2002	Prostate Cancer (PCA)	LCM BPH, PIN, PCA Cell lysate	SELDI-TOF-MS	Several small molecular mass peptides and protein (3000–5000Da) more abundant in PIN and PCA 56666 Da peak upregulated in 86% of BPH	Protein profiles from prostate cells with different disease states have discriminating differences. Pioneer study in pattern profiling
Adam BL et al, 2002	Prostate Cancer (CaP)	Serum samples BPHPCA and normal	SELDI-MS coupled with artificial intelligence learning algorithm using a nine protein mass pattern		Algorith correctly classified 96% of the samples with 83% sensitivity and, 97% specirficity and 96% predictive value. Classification system highly accurate and innovative approach for early diagnosis of PCA
Petricoin et al, 2002	Prostate	Serum	SELDI-TOF-MS, H4 Array	The proteomic pattern correctly predicted 36 of 38 patients with prostate cancer while 177 of 228 patients were correctly classified as having benign conditions.	Serum proteomic pattern analysis may be used in the future to aid clinicians so that fewer men are subjected to unnecessary biopsies.
Petricoin III et al, 2002	Prostate CaP screening (Chile clinical trial)*	Blinded Serum LCM prostate cells	SELDI-TOF-MS C16 chip Bioinformatics/pattern generation: use of genetic algorithm to obtain a pattern that best segregates between training sets of spectra from sera of BPH and CaP	Best proteomic pattern obtained from bioinformatic algorithm	Evaluated the ability to detect and discriminate BPH and CaP in men with normal or elevated PSA levels Algorithm from training sets correctly classified prostate cancer patients in 95% of cases Potential secondary screen for men who have marginally elevated PSA serum levels. Patients classified as BPH by biopsy and as CaP+ by serum proteomics pattern in a folloup study subsequently developed cancer
Qu et al, 2002	Prostate	Serum	SELDI-TOF-MS, IMAC Array	Boosted decision tree classification was used to find 12 protein peaks ranging from 3–10 kDa to differentiate prostate cancer versus non-cancer; 9 different proteins ranging fin size from 3–9 kDa were similarly selected to differentiate healthy versus benign prostatic hyperplasia (BPH) patients	Boosted decision tree analysis employed to reduce problem of overfitting the classification model and have an easy to interpret model as an outcome. In one part of the study, 100% sensitivity and specificity was achieved in classifying 197 cancer patients versus 96 healthy individuals. Greater than 90% sensitivity and specificity was also achieved in the test set for the more difficult problem of distinguishing BPH.

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TABLE 3. (*Continued*) Achievements in Cancer Biomarker Detection

Reference	Type of Cancer/ Clinical Study	Source of Biological Material	Proteomic Technology of Choice	Biomarker/ Surrogate	Significance and Potential
Adam BL et al, 2001	Prostate Cancer (CaP)	LCM Cell lysate and Serum from BPH, PIN, PCA	SELDI-MS	Pattern profile different in PIN PCA BPH and normal	Pioneer study in SELDI-MS Importance of pattern profiling for biomarker discovery
Howard et al, 2003	Lung	Serum Small lung cancer tissue sample and non small lung cancer	Isoelectric focusing (IEF) MALDI-TOF-MS and genetic algorithm analysis Protein identification by RP-HPLC/C18 column and SDS-PAGE In gel tryptic digestion Peptide mapping AntiSAA immunoblot ELISA quantitation of SAA in lung cancers vs normal	Protein expression profile Identification of Serum Amyloid A (SAA)	MALDI-TOF MS powerful tool in the search of serum biomarkers of lung cancer and in discriminating between serum from lung cancer patients from that of normal individuals Potential alternative strategy and non invasive diagnostic tool for lung cancer
Yanigisawa et al, 2003	Lung tumors	Fresh frozen lung tumor tissue	MALDI TOF Training algorithm	1600 protein picks, class prediction models ableto classify lung cancer histologies, distinguish primary tumors from metastasis and classify nodal involvement	Proteomic patterns obtained directly from small amounts of fresh frozen lung tumors tissue accurately classified and predicted histological groups as well as nodal involvements and survival in resected non small cell lung cancer
Steel et al, 2003	Hepatocellular carcinoma (HCC)	Fractionated Serum from clinically defined diagnostic groups: Active, inactive, chronic HBV and controls	2 dimensional gel electrophoresis (2D GE) tryptic fragment mass fingerprinting	Identification of C3 and isoform apolipoprotein A1	Proteomic methodologies can be used for the identification of serum biomarkers in HCC
Poon et al, 2003	Hepatocellular carcinomas	Serum	SELDI-TOF-MS, Anion Exchange Fractionation, IMAC and WCX Arrays	250 differentially regulated protein peaks detected in a 20 × 38 study were narrowed down by an Artificial Neural Network (ANN) and Significant Analysis of Microarray (SAM) data approach to 10 most significant differentiators ranging from 4.6 tp 51.2 kDa.	A potential diagnostic model was rapidly created showing good differentiation of hepatocellular carcinoma from chronic liver disease regardless of input AFP levels. ROC areas under the curve were 0.91 for all cases tested and 0.954 for cases also differentiated by AFP < 500 μg/L.
Zhou et al, 2002	Esophageal carcinoma	LCM esophageal carcinoma cells and normal epithelial cells	2D Differential in gel electrophoresis (2D-DIGE) Bioinformatic Quantitation of protein expression by 3D simulation of protein spot Protein identification by capillary HPLC/MS/MS	Annexin I (gp96) unregulated in esophageal carcinoma	DIGE new approach in comparative differential display proteomics Global quantification of protein expression between LCM patient matched cancer cells and normal cells using 2D-DIGE in combination with MS is a powerful tool for the molecular characterization of cancer progression and identification for cancer specific protein markers
Melle et al, 2003	Head and Neck	LCM procured cells	SELDI-TOF-MS and SELDI-QqTOF; H4, SAX2 Arrays	Annexin V found differentially expressed (p = 0.000029) in 57 × 44 study of tumors versus adjacent mucosa procured by LCM	Looking for a better understanding of molecular mechanisms behind tumorigenesis and tumor progression in head and neck cancer Protein expression changes between microdissected normal pharyngeal epithelium and tumor tissue (3000–5000 cells in each sampling–a reasonable number for the pathologist to excise) were analyzed by SELDI Both the mass and a rough estimate of pI of a putative marker at 35.9 kDa were determined by SELDI and this information was used to guide isolation

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TABLE 3. (*Continued*) Achievements in Cancer Biomarker Detection

Reference	Type Cancer/ Clinical Study	Source of Biological Material	Proteomic Technology of Choice	Biomarker/ Surrogate	Significance and Potential
					of the marker by 2D-PAGE; following isolation, in-gel digestion and peptide mapping was performed by SELDI-TOF-MS and a high confidence ID score for annexin V was obtained showing a rapid pathway from marker discovery to ID. Further ID verification was performed by a SELDI immunodepletion assay as well as SELDI-QqTOF partial sequencing.
Wadsworth JT, 2003	Head and Neck cancer	In press	In press	In press	In press
Review by Le Naour, 2001	Neuroblastoma (NB) Breast	Autologous Tumor cells and serum	Serological proteome analysis (SERPA) 2D-GE and serum immunoblotting	b-tubulin I and III isoform (NB) RNA binding protein regulatory subunit RS-DJ1 (breast cancer)	Occurrence of autoantibody and proteomic screening in different cancers may be useful in cancer screening and diagnosis Potential of SERPA to detect new biomarkers
Klade et al, 2001	Renal Cell Carcinoma (RCC)	Autologous Tumor cells and serum	Serological proteome analysis SERPA 2D-GE and serum immunoblotting	SM22a, CAI	
Shiwa et al, 2003	Colon	Cell Culture	SELDI-TOF-MS, H4, NPI, WCX2, SAX2, IMAC3 Arrays	One biomarker of 12 kDa. Identified as prothymosin-α by SELDI-TOF-MS and confirmed by SELDI-QqTOF	Prothymosin-α could be a potential biomarker for colon cancer adding to existing markers (CEA and CA19-9) that show relatively poor predictive value. Expression screening by SELDI-TOF-MS and on-chip "retentate chromatography" were used to rapidly develop a mini-column purification scheme to isolate enough enriched candidate 12 kDa for positive identification by SELDI peptide mapping and confirmatory SELDI-QqTOF analysis.
Nakamura et al, 2002	Acute leukemia	Cell cultures	Combination of column chromatography, 1D SDS-PAGE and SELDI-TOF-MS peptide mapping	27 interactors of ALL-1 protein, a histone methyltrasferase, were identified	The ALL-1 supercomplex of proteins is believed to be a significant player in transcriptional regulation and is involved in acute leukemia. As a functional study, 27 of more than 29 interactors believed to exist were rapidly isolated and identified. Studies of this nature may lead to the creation of diagnostically relevant interaction assays.

and for different age groups." It appears that the "high-resolution protein pattern profiling" could soon be implemented in clinical trials (NIH/NCI-FDA/CBER Clinical proteomics program http://clinicalproteomics.steem.com, http://clinicalproteomics.steem.com/pdf/proteomonitor.pdf). In fact, protein profiling of ovarian and prostate cancer could discriminate normal conditions from early-stage disease and predict (prostate study only) subsequent occurrence of disease in otherwise disease-free individuals (according to PSA by ELISA testing). Criticism regarding the reproducibility and standardization of the pattern profiles described in these and other studies are being addressed, and results are promising. Modified and more specific high-throughput mass spectrometry instrumentation and the associated software programs have dramatically improved resolution and accuracy (pattern recognition with protein identification (SELDI-QqTOF; Fig. 2 from NIH/NCI-FDA/CBER clinical proteomics program http://clinicalproteomics.steem.com).

LC/MS/MS and MudPIT technologies should also be implemented for their significantly higher sensitivity than 2D-PAGE and SELDI and more extensive coverage of the proteome for biomarker discovery. High-throughput protein arrays could also be introduced in clinical trials to screen and measure known proteins. Protein arrays are used for high-throughput detection of cytokines, chemokines, and soluble factors in the serum of renal cell carcinoma patients enrolled in a high-dose IL-2 immunotherapy clinical trial. A custom-made antibody-based platform including several lymphokines, chemokines, and growth factors was commercially developed to measure their level in crude serum. A first step in diagnostic screening of this type allows the rapid exclusion of factors irrelevant to the diagnosis of the disease and its progression or response to therapy. More importantly, these high-throughput screenings may identify clusters of proteins (signatures) descriptive of various biologic states such as response to therapy or therapy-induced toxicity. A combination of the

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TABLE 4. Common Problems with Serum/Plasma Protein Biomarker Identification

Biological Material	Problems	Potential Solution	Significance and Potential
Plasma/Serum	Material degrades rapidly at room temp; proteins release (or bind to) binding partners generating fluctuations in concentrations	Standardization of serum collection Minimize Freeze thaw cycles Aliquoting in minimal volume	Ability to make multiple assessment by a combination approaches
	Extraordinary dynamic range more than 10 orders of magnitude separate albumin and the rarest protein measured now clinically Difficult mining low abundance biomarkers due to a small number of proteins such as albumin, α2-macroglobulin, transferrin, and immunoglobulins, which represent as much as 80% of the total serum protein	Pieper et al, 2003 Largest effort to date to characterize the serum proteome using a combination of 2DGE and MS Method: COMBINATION OF 1) Immunoaffinity (subtraction chromatography (ICH) 2) Anion exchange chromatography 3) Size exclusion chromatography (SEC) 4) 2D-GE 5) MALDI 6) LC MS/MS 7) (ICH) for albumin, haptoglobin, transferrin, thrasthyretin, trypsin 1-acid glycoprotein, hemopexin, a2 macroglobulin	The serum human proteome: display of nearly 3700 chromatographically separated protein spots on 2DGE and identification of 325 distinct proteins Methods holds promise to accelerate the discovery of novel serum biomarkers
	Popular high resolution 2DGE restricted loading range Removal of albumin may remove specifically bound ligands, and the presence of non specific interaction in affinity columns may remove non targeted proteins Pre-fractionation raises issues of recovery of certain components	Wu et al, 2003 Direct analysis of plasma (unfractionated) by several LC/MS approaches Method: shotogun sequencing approach Use of HPLC coupled with MS ESI FTCR Mass spectrometer Ion trap MS/MS analysis ICAT approach Additional ref: Adkins et al, 2002{}; Anderson et al{}, 2002	Use of multidimensional analytic approaches is necessary for the analysis and identification of complex biological samples ICAT can successfully quantify different levels of proteins (low to medium)
Plasma/Serum	Biological diversity–proteomic patterns correlating with disease are very "noisy" due to wide variation in proteomic fingerprints between individuals (i.e. comparing control patients in a population study)	SELDI-TOF-MS lends itself to the biological variation problem as it can screen large numbers of samples quickly to ascertain relevant protein expression differences. Running more samples provides the most direct route to elimination of non-relevant differences within the sample cohort. SELDI-TOF-MS is one of the best technologies for addressing the protein abundance dynamic range issue. Methods of serum pre-fractionation work well with SELDI as both albumin depleted and albumin-rich fractions from a single sample can be rapidly processed in parallel (see Poon et al, 2003). Alternatively, IMAC chip chemistry has been used effectively in a single step protocol to eliminate most of the albumin signal while generating useful biomarker patterns directly (see Qu et al, 2002).	Ability to process many samples in a reasonably high throughput manner is an enabling factor in biomarker discovery. Experimental design should include not only a reasonable number of control versus disease samples, but also samples representing additional disease states to validate method specificity. Again, this means biomarker studies necessarily require the ability to analyze large sample pools rapidly in a quantitative manner.

strategies mentioned above might be the best approach for future biomarker discovery and application in the clinical setting using modern bioinformatics strategies.[51,52] The ability to identify novel targets for biotherapy using these strategies also exists.[53,54]

Clinical Opportunities

In addition, a search could be implemented for more specific biomarkers discriminating patients with early-stage or minimal disease from normal individuals: (1) multicenter collections of plasma/serum/tissue for individuals at risk of developing recurrence of malignant disease; (2) registry studies (extensive patient history correlated with transcriptional and proteomic profiling; (3) single or multicenter clinical trials could be prospectively designed to collect materials relevant to the interpretations of the disease and its response to treatment; or (4) these proteomic initiatives and consortiums could be contacted for further information and suggestions:

Proteomic web sites: proteomic initiatives, fundings, initiatives news and views, companies NCI/Cancer Diagnosis Program (CDP)

Advice and Resources for Cancer Diagnostics Researchers

http://www.cancerdiagnosis.nci.nih.gov/assessment/index.html

Cancer Molecular Analysis Project C-MAP

http://cmap.nci.nih.gov/

Academic Public/Private Partnership project (AP4)

http://grants.nih.gov/grants/guide/rfa-files/RFA-CA-04-005.html

http://deainfo.nci.nih.gov/concepts/AP4conceptU54.htm

NIH/NCI-FDA/CBER Clinical proteomics program databank http://clinicalproteomics.steem.com

SBIR and non-SBIR cooperative grants to accelerate clinical applications

http://www.nsbdc.org/assistance/sbir_sttr/

NCI/Cancer Diagnosis Program (CDP)

Advice and Resources for Cancer Diagnostics Researchers

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Table 3b. Common problems and possible solution across different sample types

Common problems/ obstacles encountered in protein identification and analysis :

- Sample integrity: Material degradation/ standardization of sample collection
- Sample quality in tumor-burdened or diseased patient (icteric, lipemic, etc.), variability of binding proteins (Ig, Albumin)
- Limited sample volume for repeat analysis
- Enormous dynamic range of expressed proteins
- Difficulty in mining low abundance proteins
- Incompatibility of data output among different platforms/instruments

Possible solutions:

- Standardization of material collection, aliquoting, no freeze thaw cycles
- Biochemical analysis of sample/enzymatic profile at the moment of collection
- Utilization of high sensitivity HPLC and MS
- Elimination of high abundance proteins by fractionation
- Enrichment of low level protein families by concentration
- High throughput MS and Protein array technology
- Utilization of internal protein controls (added to the sample)
- Automation of testing, data acquisition and analysis
- Protein chips, arrays, platforms using low sample volume for global screening
- New ligand antibody pairs for sample enrichment and array analysis
- Enhanced crossreactivity testing and epitope mapping to increase sensitivity and specificity
- Combination of different methodologies could be the future of biomarker discovery

Software standards for proteomic data integration, intelligent data approach

FIGURE 1. Common problems and possible solution across different sample types.

http://www.cancerdiagnosis.nci.nih.gov/assessment/index.html

NCI/The Early Detection Research Network: Biomarkers Developmental Laboratories (EDRN)

National network that has responsibility for the development, evaluation, and validation of biomarkers for earlier cancer detection and risk assessment

http://www3.cancer.gov/prevention/cbrg/edrn/

http://grants1.nih.gov/grants/guide/rfa-files/RFA-CA-99-007.html

Proteomonitor, the global newsweekly of proteomic technology

http://www.proteomonitor.com/index.htm; http://clinicalproteomics.steem.com/pdf/proteomonitor.pdf; http://proteomicssurf.com/forums/forumdispaly.php

Ciphergen

15,000 data points

•Low Resolution

•MALDI-TOF ions do not necessarily reflect relative abundance in sera

CL558-WCX2-B

The ABI QSTAR® Pulsar Hybrid LC/MS/MS System is a high performance hybrid quadrupole time-of-flight mass spectrometer

Higher resolution
ABI QSTAR
350,000 data points

FIGURE 2. High-performance mass spectrometry obtainable by using the SELDI system coupled with QStar (SELDI-QqTOF) (http://clinicalproteomics.steem.com).

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Means to Advance This Most Quickly

We would recommend that (1) NIH/FDA/Biotech Cooperative Grants (non-SBIR) be funded to accelerate clinical applications; (2) NIH/FDA/pharmaceutical companies provide training/upgrades; (3) develop core facilities with an array of advanced instruments/technologies available; and (4) enable sharing of results with development of an open-access multi-institutional website similar to the NCI WEB site. To realize these strategies, we recommend the following tactics: (1) constant upgrade of bioinformatics tools; (2) implementation of training systems (normal vs. disease) with large databases for SELDI bioinformatics tools for proteomic pattern diagnosis; (3) as each new patient is validated through pathologic diagnosis using retrospective or prospective data sets, its input can be added to an ever-expanding training set[21]; and (4) establish array database of normal ranges from various demographic populations to allow valid comparisons to disease states. The workshop participants suggested that a national repository for serum/plasma should be created, as well as a list of the best practices to use for serum/plasma collection. HUPO can help determine consensus standards for collection. Standardization of serum/plasma analysis needs to be generated as soon as possible by a combined effort of interested groups, including the NCI, FDA, WHO, Red Cross, and so forth. We also recommended that there be standardization and validation of techniques: (1) There should be a fixed number of freeze/thaw cycles and routine addition of protease inhibitors; (2) Chemical qualification of samples needs to be performed before analysis (total protein, lipids, enzymes, etc.); (3) Supporting validation of each protein measured should be attempted; (4) Good laboratory practices and quality control of instrument and reagents needs to be established prior to sample analysis and control trending; and (5) Eventual regulatory compliance should be initiated, as in the diagnostics industry.

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IMMUNE POLYMORPHISMS

The aim of this component of the Cancer Biometrics Workshop was to determine whether immune response gene polymorphisms are of clinical relevance in cancer and to recommend strategies for further analysis in this nascent area. At present, study of such polymorphisms does not affect strategies for patient management and treatment, due to the emerging nature of the field. The workshop aimed to critically evaluate present data and to propose definitive studies in this area. Specifically, the following question was addressed: At present, can particular immune response gene polymorphisms of clinical relevance be identified in diagnosis, prognosis, and tailoring of therapy in any specific cancers?

State of the Art

Many genes and gene families whose products play a critical role in regulating the immune response are highly polymorphic, and this polymorphism can lead to interindividual differences in antigen-specific and non-antigen-specific immune responses. In cancers in which anti-tumor immune responses occur, this polymorphism can modulate susceptibility to and/or prognosis in the malignancy concerned. Immune response gene polymorphisms may also modulate response to tumor therapy. A brief summary of polymorphisms in critical gene families, along with examples of associations with disease susceptibility/prognosis (including cancer), follows. Current and future techniques for immune polymorphisms genotyping are also considered. These summaries are not exhaustive but serve to illustrate the state of the art in this field.

Human Leukocyte Antigen (HLA) Genes

The genes encoding the HLA molecules, located within the human major histocompatibility complex (chromosome 6p21.3), are the most polymorphic within the human genome, with at least 282 HLA-A, 537 HLA-B, 135 HLA-C, 418 HLA-DRB, 24 HLA-DQA1, 55 HLA-DQB1, 24 HLA-DPA1, and 106 HLA-DPB1 alleles currently recognized (IMGT/HLA database: http://www.ebi.ac.uk/imgt/hla). The overwhelming majority of HLA polymorphisms are functional, resulting in amino acid substitutions in the peptide-binding grooves of these molecules, so determining the repertoire of processed antigenic peptides that can be presented to CD4+ and CD8+ T cells. Due to this critical role of the HLA system in regulating the immune response, combined with its exuberant polymorphism, it is not surprising that particular HLA polymorphisms have been linked to a large number of immunologically mediated diseases, including skin, gut, endocrine, and joint diseases.[1] In addition, several studies have indicated that HLA polymorphism may mediate susceptibility to both hematologic malignancies (eg, Hodgkin disease,[2] childhood acute lymphoblastic leukemia,[3,4] and chronic myeloid leukemia[5]) and non-lymphoreticular malignancies, including cervical cancer,[6] breast cancer,[7] and malignant melanoma.[8] In malignant melanoma, HLA-DQB1*0301 polymorphisms have also been reported to be associated with patient survival.[9] In addition, selective or complete loss of HLA class I expression is a frequent occurrence in many tumors, resulting in escape from T-cell immune surveillance.[10] HLA associations with malignant diseases are reviewed in more detail elsewhere.[11]

Cytokine Genes

Cytokines are generally small molecules secreted by one cell to alter the behavior of itself or another cell, generally within the hematopoietic system. Cytokines act on target cells

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by binding to specific receptors, initiating signal transduction and second messenger pathways within the target cell. Cytokines function as players in a highly complex coordinated network in which they induce or repress their own synthesis as well as that of other cytokines and cytokine receptors. Production of numerous cytokines by the cells of the immune system, in response to both antigen-specific and nonspecific stimuli, plays a critical role in the generation of both pro- and anti-inflammatory immune responses.

In recent years, many single nucleotide (SNP) and a more limited number of microsatellite polymorphisms have been detected within cytokine gene sequences, particularly within the promoter regions of these genes. Several of these polymorphisms may be associated with differential levels of transcription of the genes concerned (eg, TNFα-308 and IL-10-1082), although cell type and stimulus may also be important. Much effort has been directed toward investigating whether these polymorphisms are likely to play a role in immune-mediated diseases, and a considerable literature now exists. For example, there are reported associations between SNPs in the TNFα promoter and rheumatoid arthritis, cerebral malaria, asthma, and cardiac and renal transplant rejection. Similarly, associations between IL-10 promoter polymorphisms and systemic lupus erythematosus and asthma have been described. However, there is still considerable conflict and uncertainty in the literature with regard to many diseases, genes, and SNPs. Excellent reviews of the literature with regard to cytokine polymorphisms, relationship to gene expression, and disease associations are available, both in print and online.[12,13]

The literature with regard to cancer is small but growing rapidly. A number of studies have reported associations between TNFα and/or LTα SNPs and particular cancers, including chronic lymphocytic leukemia,[14] non-Hodgkin lymphoma,[15] and breast cancer,[16] although negative findings are reported by others.[17,18] In this context, study of IL-10 polymorphisms is of particular interest, since IL-10 has both anti-inflammatory and anti-angiogenic properties. Genotypes associated with high IL-10 production in vitro have been reported to be protective in cutaneous malignant melanoma[19] and prostate cancer,[20] while low expression genotypes are a risk factor, both for disease susceptibility and markers of disease severity/prognosis. These results are consistent with the anti-angiogenic properties of IL-10. To date, 15 separate investigations of IL-10 polymorphism in 10 different cancers have been performed, with positive associations (with high or low IL-10 expression genotypes) reported in 12 of these studies. Results from these studies are summarized in Table 5.

Killer Immunoglobulin-like Receptor (KIR) Genes

KIRs are expressed on the surface of NK cells and also certain T-cell subsets. KIRs may be either inhibitory or activating, with inhibitory and some activating KIRs recognizing HLA class I as ligands (principally HLA-B and C epitopes). As yet, the ligands for other activating KIRs are unknown. Genes encoding KIR are located on chromosome 19q13.42, within the leukocyte receptor cluster (LRC). The KIR gene cluster consists of up to 17 expressed genes. Diversity results from differing gene copy number and content between individuals and from allelic variation of individual genes (up to 12 alleles for some loci). There is also variation in expression of individual KIR genes, both between individuals and between NK cell clones within the same individual, although this phenomenon is still poorly understood.[34,35] Population genetic studies are underway to more fully characterize KIR haplotypes and polymorphism in different human populations. Due to the role of KIR in regulating NK responses, combined with a rapidly expanding knowledge of KIR diversity and polymorphism, there is considerable interest in determining whether KIR polymorphism plays a role in determining disease susceptibility and/or disease progression. It has been shown that possession of KIR3DS1 in combination with HLA-B alleles encoding isoleucine at position 80 is associated with delayed onset of AIDS,[36] while KIR2DS2 is involved in the development of rheumatoid vasculitis and KIR2DS1 with psoriatic arthritis.[37] There are no reports (to date) of KIR associations in cancer, although patient/donor KIR mismatching in allogeneic bone marrow transplantation may be beneficial, via a contribution to the GvL effect and by reduction of GvHD.[38] In addition, tumors develop sophisticated patterns of loss of HLA class I expression, so as to escape T-cell immune surveillance while maintaining inhibition of NK cell responses, mediated by HLA class I/KIR interactions.

Leukocyte FCγR Genes

Leukocyte Fcγ receptors (FcγRs) confer potent cellular effector functions to the specificity of IgG. FcγR-induced leukocyte functions, including antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, superoxide generation, degranulation, cytokine production, and regulation of antibody production, are essential for host defense and immune regulation.[39] The efficacy of such IgG-induced FcγR functions shows interindividual heterogeneity due to genetic polymorphisms. At least three functional amino acid substitutions have been described (FcγRIIa-131 H/R [CD32a], IIIa-158V/F [CD16a], and IIIb-NA1/NA2 [CD16b]), with associated modulation of immune function.[39] A number of additional, apparently nonfunctional, polymorphisms have been described with specific interest for polymorphisms in the inhibitory receptor FcγRIIb, which could possibly alter the balance between activating and inhibitory signals.[39] FcγR polymorphisms have been associated with increased susceptibility to or severity of disease of several infectious (meningoccal disease) and autoimmune diseases (eg, systemic lupus erythematosus, Wegener's granulomatosis, rheumatoid arthritis [RA], Guillain-Barré syndrome [GBS]).[39,40]

Therapeutic effectiveness of monoclonal antibodies and adverse reactions have been shown to depend largely on antibody isotype, antibody affinity and epitope recognition, and interaction with FcγR. Monoclonal antibodies for the treatment of cancer represent mostly chimeric or humanized IgG1 monoclonal antibodies.[41] Therapeutic efficacy of these antibodies involves several mechanisms including FcγR-induced ADCC by macrophages, monocytes, and NK cells.[42] Two studies demonstrate the importance of FcγR polymorphisms for therapeutic responses to the anti-CD20 IgG1 monoclonal antibody rituximab (Rituxan). Patients homozygous for FcγRIIIa-158V, the FcγRIIIa allotype most efficiently

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TABLE 5. Immune Polymorphisms in Cancer

Disease	IL–10 polymorphism	Cases	Controls	Association	Genotype, allele or haplotype	References
Cutaneous malignant melonoma	−1082, −819, −592	153	158	Susceptibility, advanced stage of disease, greater tumor thickness	−1082 AA	Howell et al, 2001[19]
				Greater tumor thickness	ACC/ACC, ACC/ATA, ATA/ATA	Howell et al, 2001[19]
				Noninvasive growth phase	−1082 GG	Howell et al, 2001[19]
				Noninvasive growth phase	−GCC/GCC	Howell et al, 2001[19]
Cutaneous malignant melonoma	−1082, −819, −592	42	48	Survival (shorter)	ACC/ATA	Martinez-Escribano et al, 2002[21]
Prostate cancer	−1082	247	263	Susceptibility	−1082 AA	McCarron et al, 2002[20]
Breast cancer	−1082	144	263	No	—	Howell et al, 2003[22]
Breast cancer	−1082	125	100	Susceptibility	−1082 AA	Giordani et al, in press[23]
Cervical cancer	−1082	77	69	Susceptibility	−1082 AG	Stanczuk et al, 2001[24]
Cervical cancer	−1082, −819, −592	144	179	No	—	Roh et al, 2002[25]
Gastric carcinoma	−1082, −819, −592	220	230	Susceptibility, advanced stage	GCC (1 or 2 copies)	Wu et al, 2003[26]
Gastric carcinoma	−1082	150	220	Association with EBV-negative gastric carcinoma	−1082 G allele	Wu et al, 2002[27]
Gastric carcinoma	−1082, −819, −592	188	212	Susceptibility (non cardia gastric cancer)	ATA haplotype	El-Omar et al, 2003[28]
Squamous cell carcinoma of skin (post renal transplant)	−1082, −819, −592	70	70	Susceptibility	GCC haplotype	
				Protection	ATA haplotype	Alamartine et al, 2003[29]
Multiple myeloma	IL-10G, IL-10R microsatellites	73	109	Susceptibility	IL-10 G 136/136, IL-10 R 112/114	
				Protection	IL-10 R 114/116	Zheng et al, 2001[30]
Myelodysplasia Acute myeloid leukemia	−1082, −819, −592	150	up to 1000	No	—	Gowans et al, 2002[31]
Non-Hodgkin's lymphoma	−1082, −819, −592	126	302	Susceptibility to aggressive disease	−1082 AA, ATA, ACC haplotypes	Cunningham et al, 2003[32]
Acute lymphoblastic leukemia	−1082	135	—	Protection from poor response to prednisone treatment	−1082 GG	Lauten et al, 2002[33]

triggered by IgG1, showed increased response rates (RR) to rituximab compared to other genotypes after 2 months.[43,44] In addition, a combination of FcγRIIa-131H/H and FcγRIIIa-158V/V were independent predictive factors for long-term RR (6 months to 1 year).[43] Interestingly, higher RR could be translated to significantly longer remissions.[43] Increasing therapeutic dosage of the antibody up to 100-fold may compensate for unfavorable FcγR genotypes.[45] Upfront knowledge of an individual's FcγR genotype could therefore be important for increasing therapeutic effectiveness.

Other Immune Response Genes

A number of polymorphisms have been identified in other genes of immunologic relevance, including Toll-like receptors, CD-14, adhesion molecules, and chemokine genes. Studies of these gene polymorphisms in disease development are in their infancy. Polymorphism of the TLR-4 gene influences atherogenesis[46] and septic shock following gram-negative bacterial infection.[47] Thus far, these polymorphisms have not been studied in cancer.

Genotyping Methodology: State of the Art

A wide range of methods are currently in use for genotyping SNPs and polymorphisms in genes with multiple polymorphic sites. All of these methods are based upon the use of PCR. Most methods can be considered "traditional" and are suited to genotyping relatively small numbers of samples for one or a few SNPs. The strengths and weaknesses of each method are briefly considered below.

1. ARMS (amplification refractory mutation system) PCR or PCR-SSP (sequence-specific primers). Applicable to SNP genotyping and widely used for HLA typing in clinical laboratories, especially for "low" or "medium" resolution typing of closely related allele groups. Rapid, but most suited to low-throughput work. Can be multiplexed, but this requires additional optimization.
2. PCR-SSOP (sequence-specific oligonucleotide probe). Can be used for typing both SNPs and multi-allelic systems such as HLA. Suitable for all levels of allelic resolution, particularly for batch analysis of large numbers of samples. Commercially available in "reverse-SSOP" format for rapid typing of small numbers of samples.
3. PCR-RFLP (restriction fragment length polymorphism). Useful for SNP genotyping but can be used for some HLA analysis. Useful for low to moderate throughput.
4. Real-time liquid phase fluorescent PCR (eg, TaqMan and Light Cycler systems). Good for medium- to high-resolution SNP genotyping. Some utility for HLA. Ease of optimization, but more expensive for one-off small studies.

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5. PCR-SSCP (single-strand conformational polymorphism). Good for screening for new mutations.
6. Sequence-based typing. Confirmation of new mutations/polymorphisms. Can be used for allelic-level HLA typing.
7. Double-strand DNA conformation methods such as reference-strand conformation analysis (RSCA). Commercially available systems for HLA typing.
8. Oligonucleotide microarray. Simultaneous SSOP-type approach for many SNPs and multiallelic loci. This is undoubtedly the preferred approach for anlysis of multiple SNPs in genes influencing common or related pathways.

Some of these methods require considerably more DNA than others (eg, ARMS-PCR vs. real-time PCR). Amplicon lengths also vary. All methods work well on peripheral blood-derived DNA, but methods based on a short amplicon length work best when using small amounts of variably degraded DNA, such as that derived from archival, fixed biopsy tissues.

Limitations and Opportunities Arising From Existing Studies of Immune Response Gene Polymorphisms and Cancer

As yet, only a small literature exists in this field, and few consensus associations with therapeutically useful indicators—such as markers of prognosis or response to therapy—have emerged. For many polymorphisms, only a single study in a given cancer has been performed. Even when this is not the case, most studies are based on small numbers of cases and controls, which may be population rather than matched controls. Very often, such studies have been purely of a case-control nature, with markers of prognosis and/or disease-free survival not examined. In addition, very often only a single SNP per gene has been examined, making it difficult to exclude a role for polymorphisms in the genes concerned in cancer susceptibility/prognosis. The illustrative results for IL-10 polymorphisms and cancer given in Table 1 serve to illustrate these points. However, it should be acknowledged that one or two studies exceeding a few hundred cases and controls have been performed, such as the international collaborative investigation of the role of HLA-DPB1 polymorphism in Hodgkin's disease[2] and the role of HLA class I polymorphisms in chronic myeloid leukemia.[5] Nevertheless, despite these severe limitations of most published studies, the preliminary literature does indicate that definitive studies of selected immune polymorphisms are indicated in selected cancers, with a robust study design.

Workshop Recommendations

A definitive study of selected immune response gene polymorphisms should be undertaken in selected cancers such as cutaneous malignant melanoma, breast cancer, and/or childhood leukaemia. This will require the following:

1. Collection of peripheral blood DNA samples from sufficient cases of cancer in question (ideally 20,000, but upwards of 5,000 would still constitute a major advance)
2. Collection of appropriate control samples
3. Selection of SNPs to allow comparisons with gene expression and constructed haplotypes
4. Emerging genotyping technologies will facilitate definitive, comprehensive studies
5. Immunogenetic studies must be integrated with gene expression and proteomics
6. Collection of definitive clinical and pathological data, with full follow-up, including periodic assessment of therapeutic responses, must be an integral part of the study.

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HIGH CONTENT SCREENING USING FLOW AND IMAGING CYTOMETRY

In cancer biotherapy, the identification of both immune and nonimmune cell parameters in the peripheral blood that predict response to biologic treatments is of major importance. The utility of evaluation of cells in the peripheral blood in disease is perhaps best exemplified in the setting of HIV and AIDS, where enumeration of CD4+ T cells is reflected as integrants of the state of the disease, especially when applied in combination with viral load enumeration. This has enabled substantial progress in the testing of novel therapeutics. Monitoring of CD4+ T-cell improvement and reduction in viral load is used as a means to modify treatments and serve as surrogates in the setting of novel mono- or combination therapies. Furthermore, this has allowed the identification of substantial changes in peripheral blood cells, promoting the identification of effective agents and the more rapid licensing of effective therapeutics. These surrogates of response to therapy/survival/time to progression are not yet identified in the setting of cancer. Although many studies have evaluated the nature and quality of various cells at the tumor site (T cells, macrophages, dendritic cells, NK cells, B cells, granulocytes) as well as within the peripheral blood, a thoughtful strategy to integrate enumeration and functional/phenotypic assays has yet to be of similar utility. These strategies may be one of the newest approaches for identification of cancer biometrics useful in novel approaches to cancer care.[1]

State of the Art

Traditionally, pretreatment evaluations in biologic treatment studies in cancer patients have included studies of immune cell subsets by flow cytometry. The results are reported in percentages that reflect relative changes in lymphocyte subsets from baseline in serial monitoring or from normal controls in cross-sectional studies. There are various other lymphocyte attributes that have been shown to be altered in tumor patients during disease progression or therapy using a number of different assays (Table 6). However, these assays have not been widely applied to the clinical assessment of patients with cancer.

T Cells

Untreated patients with cancer are not generally considered to be leukopenic, yet low absolute counts of T cells as well as T-cell subset changes have been documented in cancer patients. Further, the imbalance in T-cell subsets is

TABLE 6. T-Cell Parameters in Peripheral Blood That Are Frequently Altered in Tumor Patients

Low T-cell frequencies
T-cell subset changes (CD4+, naive T cells decreased)
Decreased ζ-chain expression
Increased apoptosis (CD95, Annexin V)
Cytokine profiles
Memory T-cell function
Treg (CD4+CD25+)
Tumor-specific T-cell responses

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important, as it appears to influence survival[2] and may have a prognostic value. Parameters of general T-cell dysfunction, including downregulated T-cell receptor (TCR) zeta-chain expression,[3] enhanced expression of apoptosis markers CD95 and annexin binding,[4] and altered (T2 shift) or impaired production of cytokines[5,6] can be frequently detected in cancer patients. Evidence is available suggesting that antigen-specific T-cell responses in the setting of cancer are impaired. Nevertheless, the presence of specific T-cell responses to nominal tumor antigens and cultured tumor cell targets has been confirmed in patients with various malignancies.[7–9] Preexisting T-cell responses to tumor-specific epitopes can be detected in some patients with cancer using tetramer-based or cytokine-based technologies.[10] Correlations between the presence and frequency of tumor-antigen-specific T cells and clinical findings are beginning to emerge in patients with melanoma, colorectal cancer, renal cell cancer, and chronic lymphocytic leukemia.[10–12]

Dendritic Cells

Several studies have reported that dendritic cell (DC) defects occur in cancer patients. Specific changes have included diminished expression of MHC class II molecules, decreased expression of costimulatory molecules, including CD80 and CD86, defective maturation, decreased ability to migrate out of tumor sites to regional lymph nodes, and DC apoptosis in the tumor microenvironment.[13] A preponderance of plasmacytoid DCs within breast cancer, associated with worst prognosis, has been suggested (Lebeque S, personal communication) and demonstrated in head and neck carcinoma.[14]

NK Cells, Other Leukocytes

There is substantial literature correlating peripheral NK cytolytic activity in the blood of patients with cancer to outcome/prognosis.[15–18] Improved outcome in patients with hepatocellular carcinoma, gastric carcinoma, colorectal cancer, and multiple myleloma has been associated with increased numbers and enhanced NK cytolytic activity and number in the peripheral blood. The elusive role of the NKT cell has been difficult to discern, but a specific defect in ligand-dependent gamma-interferon production has been noted, correlating with transition to myeloma.[19] Neutrophil numbers, phenotype, and function have not been carefully assessed. Changes in platelet number and function have been reported only anecdotally. Circulating endothelial cells and other progenitor cells are being explored in patients with cancer, but so far no convincing data exist linking the presence or frequency of these cells to clinical findings.

Strategies and Context Suitable for Immediate Application

Few parameters have been identified so far for which an association with a response to biologic therapy can be convincingly shown. However, with the advent of quantitation multicolor flow cytometry, it is expected that more meaningful correlations will emerge. Absolute numbers of T-cell subsets might be measured in whole blood, using commercially available reagents in single-platform, flow cytometry-based techniques.[20] As recently illustrated, absolute numbers of T-cell subsets provide substantial and informative data with a prognostic or survival value.[2] Absolute numbers of T-cell subsets, not their percentages, are meaningful in establishing correlations with disease progression or prognosis.[20] Probably the best-studied parameter is the analysis of ζ-chain expression, which was shown to be associated with response to IL-2 therapy in patients with ovarian cancer[21] as well as metastatic melanoma treated with histamine dichloride and IL-2.[22] The restoration of ζ-chain expression was associated with response to a PSA vaccine in patients with prostate carcinoma[23] as well as to cytokine therapy in patients with melanoma and renal cell cancer.[3,24,25] The TCR ζ-chain expression can be now measured by quantitative flow cytometry and the results reported in the MESF unit read off the standard curve established with fluorescent beads.

The presence and absolute numbers of regulatory CD4+CD25+ T cells at the tumor site and in the peripheral circulation of patients with cancer can be quantitated by flow cytometry and linked to the overall level of immune dysfunction in patients with cancer. Flow cytometry-based assays for functional properties of these regulatory cells are in the development and will shortly replace the mixing or co-incubation assays, which require large numbers of cells. Readily available today are multiplex assays for simultaneous detection of multiple cytokines and chemokines, allow for profiling of these mediators in minimal volumes of sera or body fluids of patients with cancer. Similar in principle to flow cytometry, these assays, based on the use of Ab-coated multicolored beads, allow for the detection of Th1 versus Th2 bias in disease. Newer flow cytometry-based assays make it possible to combine the detection of phenotypic and functional attributes at a single-cell level. In this category are currently available assays combining tetramer staining with intra-cytoplasmic cytokine detection or with perforin/Granzyme B expression. In this manner, tumor antigen-specific T cells can be enumerated and their functional potential discerned. These single-cell assays have been particularly useful in evaluating the frequencies of tumor-specific T cells after vaccination therapies.[26]

The list of currently available but not routinely used assays with a potential to provide meaningful information about the immune status of patients with cancer includes:

1. Annexin V binding to T cells or T-cell subsets, which do not simultaneously stain with propidium iodide (PI-), gives a measure of spontaneously apoptotic lymphocytes in the peripheral circulation.[27] Annexin V binding to T cells has been linked with poor prognosis in patients with HNC and melanoma.[3,27] This assay must be performed with freshly harvested cells.
2. Quantification of naïve, memory, and effector T-cell subsets (eg, CD3+CD45RA+CCR7+, CD3+CD45RA$^-$CCR7+ and CD3+CD45RA$^-$CCR7$^-$) in the patient's circulation by multicolor flow cytometry[28]
3. NFκB p65 assays to look for defects in translocation of NFκB to the nucleus[29]
4. A variety of cytotoxicity assays adapted for flow cytometry (as opposed to ^{51}Cr-release assays) to measure NK activity and numbers (if K562 is a target) or functions of activated

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T cells with autologous HLA-restricted targets. In this regard, inclusion of cytotoxicity assays in the flow repertoire is considered important. In patients with metastatic colorectal cancer treated with the monoclonal antibody 17-1A, the robust pretreatment NK cell cytolytic activity against K562 targets was identified as a strong prognostic factor for response to therapy.[30]

Future immunotherapy therapy trials should be designed to determine more precisely whether the general T-cell dysfunction observed in many patients with cancer correlates with response to therapy. As indicated, TCR-associated ζ-chain downregulation as a general marker for T-cell dysfunction is an assay that is currently validated and could be immediately included in monitoring of clinical trials. The detection of the expression of apoptosis markers, CD95 and annexin V, is reliable only in fresh blood samples and therefore is difficult to apply in clinical trials. T-cell phenotypes including T-cell differentiation subsets and regulatory T cells as well as T-cell cytokine profiles are other parameters that reflect interactions of T cells with the tumor and are reasonably easy to reliably perform in the setting of clinical trials. Assessments of clonal T-cell expansion by Immunoscope and TcLandscape Technology, which define T-cell receptor repertoire, are likely to become more important in the near future for the analysis of epitope spreading following antitumor vaccine administrations.

In monitoring of antitumor therapies, the crucial point is the analysis of an association between the presence and quality of tumor immunity and clinical responses to treatment. Since autologous tumor cells are usually not available for T-cell monitoring, T-cell responses to panels of HLA-matched allogeneic cell lines may be analyzed, as demonstrated for patients with melanoma. Such responses were detected in a substantial proportion of patients even with stage IV disease.[31] Alternatively, T-cell responses to known epitopes presented on HLA-matched antigen-presenting cells (eg, T2 cells) could be determined, which is much easier to standardize and probably equally informative. These studies require HLA typing of patients. Sensitivity of the assays and specificity controls are important issues for study design using these assays.

In antigen-specific vaccination, the induction of vaccine-specific T-cell responses is the primary goal of clinical trials. There is some evidence currently from vaccination studies with peptides, peptide-loaded DCs, or heat shock proteins that the frequencies of vaccine-induced T cells correlate with clinical responses.[32] A preexisting T-cell response to the vaccine peptide may be a negative predictor for immune responsiveness, since preexisting immunity to NY-ESO or tyrosinase could not be boosted in two small trials.[9,33] Thus, the strategies for determining the frequency of tumor antigen-specific T cells in the circulation of vaccinated patients with cancer appear to be providing the expected results, perhaps as a result of world-wide efforts to optimize and standardize these technologies.

Most Promising Opportunities

More recently developed techniques allowing for the simultaneous analysis of multiple targets in signaling pathways in T cells by flow cytometry[34] and the use of high content screening,[35] which is based on automated, multicolor fluorescence imaging of arrays of treated cells, are of great interest for biomarker identification, target validation, and target and cell function research.

Using antibodies specific for phosphorylated targets of kinases, the simultaneous measurement of the activation state of numerous targets can be analyzed on a single-cell level by intracellular flow cytometry. This approach will be useful to study signal transduction pathways in T cells and other cells. Following T-cell receptor activation, the differential activation of T-cell receptor downstream events was shown.[34] Also, altered signal transduction in leukemia cells in response to cytokines could be demonstrated and was shown to be associated with response to therapy.[36–38]

Complementary to multicolor fluorescence flow cytometry will be the use of multicolor high content screening, where arrays of cells adherent to substrates can be analyzed for phenotype and phenotypic response to small molecule and biologic treatments.[35] It is possible to screen the phenotypes of a million cells automatically, analyzing and archiving the data on more than a hundred morphometric parameters and up to eight fluorescence-based cellular parameters in less than 10 minutes. Specific cellular profile information can be collected based on stage of cell cycle, apoptosis, specific transcription factor activation, receptor binding, receptor internalization, cell movements, cell spreading, and organelle function, to name a few, in either fixed or living cells. These "large-scale" cellular profile data sets require the same large-scale biology data visualization tools such as cluster analyses and heat maps as have been applied to genomic and proteomic screens. Data mining of the large data sets from pathway mapping and target function studies from both flow cytometry and high content screening will also yield important new information and knowledge of cell and cell constituent functions.[35]

Practical Issues/Recommendations to Advance This Area Most Quickly

Surveys of tumor immunotherapy trials conducted post hoc or in limited single-institution trials have suggested the utility of few parameters in prediciting clinical or immunologic response to therapy.[39] This includes absolute counts of T cells, TCR ζ chain expression, NK cell cytotoxicity, and in the setting of antigen-specific vaccination, the presence and frequency of peptide-specific T cells. The current technology to assess T-cell phenotypes, T-cell receptor zeta chain expression, and antigen-specific T cells (ELISPOT assay, intracellular cytokine staining, tetramer staining) has been established in several specialized laboratories.[26] Assessment of NK cytolytic activity is difficult to routinely measure using using ^{51}Cr release; however, this assay may be substituted by more recent functional flow cytometry and high content screening assays for apoptosis. Future efforts should concentrate on standardization and validation of cellular assays as well as the implementation of quality control and quality assurance programs. New technologies including phosphoproteins as well as other intracellular targets studied by flow cytometry to identify discrete alterations in signal transduction in activated immune cells and the assessment of biomarkers and biomarker function with high content screening should be

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rapidly developed and validated for implementation in future biotherapy trials. Phenotypic and functional profiling of T-cell or other hematopoietic cell subsets is likely to provide a more comprehensive view of the molecular changes that accompany therapy than that available today.

At this time, we recommend that all patients in biotherapy studies have assessed at baseline and following therapy T-cell counts (total, CD4, CD8) and T-cell receptor ζ-chain expression by flow cytometry. As soon as the assessment of NK cytolytic activity can be reliably performed by functional flow cytometry, this assay should be implemented in clinical trials. In antigen-specific vaccination, not only the frequency of vaccine-specific T-cell responses before and after vaccination but also the analysis of the differentiation stage and cytotoxic factor phenotype may provide useful information. A central GLP laboratory specializing in immune monitoring of clinical trials has distinct advantages over "in house" ad hoc efforts, and it is recommended that future monitoring be directed to such laboratories.

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IMMUNOHISTOCHEMISTRY AND TISSUE MICROARRAYS

The rate of discovery of new genes involved in cancer and other diseases has increased quickly. The demand for analyses of these new genes in diseased tissues, especially human tumors, has grown at the same pace. To identify the most significant ones among all the emerging candidate cancer genes, it is often necessary to analyze a high number of genes in a high number of well-characterized tumors. Hundreds of tumors must be analyzed for each gene to generate statistically meaningful results. This leads to a massive workload in involved laboratories. Moreover, the analysis of multiple genes results in a critical loss of precious tissue material, since the number of conventional tissue sections that can be taken from a tumor block does usually not exceed 200 to 300. The tissue microarray (TMA) technology significantly facilitates and accelerates tissue analyses by in situ technologies. In this method, minute tissue cylinders (diameter 0.6–3 mm) are removed from hundreds of different primary "donor" tumor blocks and subsequently brought into empty "recipient" paraffin blocks. Sections from such array blocks can then be used for simultaneous in situ analysis of hundreds to thousands of primary tumors on the DNA, RNA, and protein level. Immunohistochemistry (IHC) is the method most frequently used in TMA studies. Although the method is widely used both in a diagnostic setting and for research, there are many difficulties and unsolved problems related to this method, which is heavily dependent on the quality and epitope preservation of the examined tissue.

State of the Art

The current state of the art for IHC should be viewed from two perspectives: clinical practice and research. In clinical practice, monochromatic staining, either immunoperoxidase or immunofluorescence, on frozen or paraffin sections, applied on a case-by-case basis is current best practice. These materials are analyzed and interpreted visually by experts and the results presented as a grading of staining intensity or relative area stained. In the research setting, single-, dual-, or multiple-color staining is used and digital imaging with computer-assisted or automatic image analysis is applied to provide semi-quantitative data. Research application is still largely tissue by tissue, but TMAs are receiving rapidly growing attention. Initially criticized for the small size of tissues arrayed per tumor, more than 100 papers have now strongly confirmed the utility of the method. At least 20 studies have addressed theis question of representativity, comparing IHC findings on TMAs with the corresponding traditional "large" sections.[1–9] Most of these studies reported a high level of concordance of results and concluded that inclusion more than one tissue core per donor block further increases the concordance. For example, Camp et al[1] studied expression of ER, PR, and Her2 in 2 to 10 tissue cores obtained from the same donor blocks in a set of 38 invasive breast carcinomas. They found that analysis of two cores was sufficient to obtain identical results as compared to the corresponding whole tissue sections in 95% of cases; 99% concordance was reached if four cores were analyzed, and analysis of additional cores did not result in a significant further increase of concordance. However, all these studies were based on the assumption that classical large sections, the current gold standard for molecular tumor tissue analysis, are representative of the whole tumor. This may not be entirely correct. A comparison of the volumes of an entire tumor, the tumor tissue located on a classical large section, and the tissue volume of a TMA spot suggests that the representativity problem may be about 100 to 1,000 times greater between the entire tumor and a "large" section than between a TMA sample and a "large" section. TMAs have recently become more available both from academic and from commercial sources. Recommended quality criteria for commercial TMAs include the availability of information on tissue fixation, a certificate for retention of antigenicity, as well as data on the level of patient consent. Facilitating application of IHC to TMA is the growing availability of specialized relational databases specifically designed to deal with mixed format data—that is, images and text will facilitate the handling of large data sets resulting from large-scale application of IHC to TMA.

Recommendations for Current Clinical Application

In current clinical practice, IHC can be used to help make an estimated overall prognosis, to help define the appropriate level of aggressiveness of therapy, and to predict or define an individual patient's response to therapy. Independent of therapy, lineage and functional markers can be used prognostically—for instance, to forecast survival. Prospective to therapy, phenotypic analysis of tumor cell lineage and/or the presence of the "specific" target on tumor or stroma cells can be used to determine the suitability of a given treatment scheme. Finally, retrospective to therapy, application of IHC expands to include analysis of host cell infiltrates for cell lineage (eg, NK and T cells) and functional markers as well as functional markers on tumor cells (eg, markers of apoptosis or cell cycling). Perhaps the greatest importance of TMAs in clinical applications is the possibility to array tissues collected within clinical trials. Storing pretherapeutic patient tissue in a TMA format will eventually enable researchers to execute extensive experiments to search for biomarkers predicting response to therapy.

In a research setting, IHC plays an important role in identification and mapping novel targets and in setting inclusion and exclusion criteria for clinical trials; it may even

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be useful in retrospective stratification of patients in an effort to identify subpopulations who are more (or less) responsive to a mode of therapy. In this setting, IHC coupled with TMA is a particularly powerful tool. As to yet, TMAs have mostly been used for cancer research, but there are also many applications in other research fields. Depending on the aim of a particular analysis, the currently available oncology TMAs can be divided into prevalence TMAs, progression TMAs, prognostic TMAs, and TMAs composed of experimental tissues. Prevalence TMAs are assembled from tumor samples of one or several types without attached clinicopathologic information. These TMAs are useful to determine the prevalence of a given alteration in tumor entities of interest. A typical example of a prevalence TMA has been published by Schraml et al.[6] The TMA containing 4,788 different samples from 130 different tumor types has been used for the analysis of multiple different markers on the DNA and protein level, including FISH and IHC analysis of cyclin E amplification and overexpression. Progression TMAs contain samples of different stages of one particular tumor type. They are instrumental to discover associations between tumor genotype and phenotype. Prognosis TMAs contain samples from tumors with available clinical follow-up data. They represent a fast and reliable platform for the evaluation of the clinical importance of newly detected disease-related genes. Validation studies using prognosis TMAs readily reproduced all established associations between molecular findings and clinical outcome. For example, significant associations were found between estrogen or progesterone expression[7] or HER-2 alterations[8] and survival in breast cancer patients, between vimentin expression and prognosis in kidney cancer,[9] and between Ki67 labeling index and prognosis in urinary bladder cancer.[2,7] Experimental TMAs may be constructed from tissues like cell lines,[10,11] xenografts,[12] or other cells or tissues.[13,14]

Areas for Future Advances

Several areas offer opportunities for improvements. These are listed for the field of IHC in Table 7. The problem of inhomogeneous fixation could be overcome by using unfixed tissues, employing better fixatives or existing fixatives in a more standardized way, or perhaps also more potent methods for antigen retrieval in formalin-fixed tissues. The use of more defined standards is another possible improvement. Improved antigen detection and interpretation could, for example, be achieved by dual labeling systems with or without the use of fluorescent techniques. Finally, the use of large TMAs will lead to a huge number of stained tissue samples, raising the issue of automated slide reading and informatics solutions.

TABLE 7. IHC Advancement Strategies

- Fluorescent techniques
  – New fluorochromes
- Multicolor labeling
- "Tetramers" for antigen-specific T cells
- Confocal imaging/microfluorimetry
- Morphometry
- Antigen retrieval
  – Physical vs. chemical
- Novel fixation
- Standardization
  – Reagents, control tissues, analysis

Validation Data

The major needs for TMA users would include the availability of more and better TMAs as well as of tools enabling precise analysis of stainings and a better understanding of complex TMA data and their combined relationships with clinical or pathologic features. Digital imaging and sophisticated database technologies are thus equally important for large-scale TMA applications. The general desire for better-quality TMAs may partly reflect the fact that shortcomings of IHC become much more visible on TMAs than they did on large sections. Considerable day-to-day variations can occur under seemingly identical conditions. Such problems can be caused by poorly controlled minor experimental parameters. For example, loss of antigenicity due to slide aging may start as early as 2 weeks after cutting TMA sections. Proof is lacking that suggested preventive strategies such as freezing slides or covering them with paraffin can prevent antigen decay. Difficulties with reproducibility of experiments emphasize the need for standardized controls on TMA sections (eg, arrayed "NCI 60" cell lines).

Ways to Facilitate Advances

During the workshop there was a consensus on a number of specific recommendations for ways to advance the field. For TMAs, one of the most burning demands is still a better availability of sections. Therefore, funding would be needed for arraying precious tissue resources. This especially applies to clinical trials, which by default lead to the best possible tissue collections. Also it will be important to provide appropriate reimbursement to providers of TMAs. Funding opportunities for TMA generation might be greatly enhanced if the FDA would clarify its position on data obtained from TMAs. A clear statement that TMA results are acceptable for FDA submissions might trigger more investments in this area from pharmaceutical companies. One major concern is how valuable TMAs should be stored, as decay of immunoreactivity in the first weeks after cutting sections was recognized as a major problem. For such non-high-tech research areas, it will also be important that sufficient public funding is allocated. These recommendations made in the field of IHC are listed in Table 8.

TABLE 8. Specific Recommendations for Advancing Immunohistochemistry/Tissue Microarray

- Take new look into fixation chemistry
- Systematic look at antigen retrieval
- Use arrays made up of "NCI 60" cell lines as a standard
- Use of both positive and negative control tissues
- Use adequate number of replicates
- iSBTc to establish and maintain databases

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ASSESSING IMMUNE INFILTRATE AND NECROSIS IN TUMORS

State of the Art

The presence of inflammatory cells within cancer has been described for quite some time by pathologists. Generally, improved outcome is associated with their presence in various epithelial neoplasms. The rapid evolution of molecular technology[1–6] and novel histochemical markers and tissue microarrays[2,7–9] provides the opportunity to establish a more effective means to study various forms of cancer. Much of the controversy in cancer diagnosis and pathologic assessment of prognosis lies in the application of these techniques in concert with other molecular tools, including DNA microarrays,[1,3,5] expression of histochemically defined cytokines,[10–16] integrated enzyme activity,[17,18] proangiogenic factors,[19,20] and oncogene products,[21–23] and correlating this with clinical relevance. Our group critically reviewed the current status of immunohistochemistry on specific tissue applications and correlates, including evaluation of tumor, lymph nodes, bone marrow aspirates, or peripheral blood. Further work is needed to establish which immune infiltrates should be routinely measured and in which settings. The appropriate sample size for such assays that can be validated in retrospective and prospective clinical studies needs to be determined.

Assessment in Prospective and Retrospective Clinical Trials

These technologies have increased the clinical trialist's ability to investigate complex molecular interactions that occur within clinically apparent cancers, to assess their presence during drug development in the context of clinical trials, and potentially to identify genetic and immunologic correlates with drug therapy. Repeated biopsies and aspirates of tumors, however, are not always feasible, they carry increased cost, and they have yet to demonstrably accelerate drug development. It may be necessary to convince third-party payers, regulatory groups, and the cooperative trials operations that such information is critical to the assessment of patients with established disease and contributes to cost-effective management and assessment of novel therapeutics. Development of high-throughput and efficient processes that can handle the information obtained during and prior to therapy must be carried out. These immunohistochemical strategies represent perhaps the oldest of the various approaches for identification of cancer biometrics, useful in novel approaches to cancer care. A new vision of molecular targets combining the strengths of conventional pattern recognition, the recognition of the critical interaction of inflammatory cells within the cancer, and development of new antibodies recognizing novel immune and tumor expressed proteins is required.

T Cells

Means to quantify the presence of T cells within lesions or at the periphery of tumor can now be done. The evaluation of T-cell subsets and assessment of general T-cell function/dysfunction in the tissue, means to identify how frequent certain T-cell defects can be detected in a certain tumor biopsy, and whether they are dependent on the disease stage are now possible in most tumors. CD3+ tumor-infiltrating T cells are detected within tumor-cell islets (intratumoral T cells) in ovarian cancer,[24] testicular seminomas,[25,26] esophageal cancer,[27] gastric cancer,[28,29] colorectal carcinoma,[30–38] prostate cancer,[39–42] and head and neck cancer.[43–46] Several retrospective studies have shown that the presence of tumor-infiltrating $CD3^+$ or $CD8^+$ T cells correlates with improved clinical outcome in colorectal,[37,38,47] lung,[48] breast,[49] and prostate cancer,[50] as well as vertical phase growth melanoma.[51] Recent work has highlighted the significance of intratumoral T cells—that is, T cells penetrating tumor islets as opposed to the surrounding stroma. Typically the presence of intratumoral $CD8^+$ T-cell infiltration more than peritumoral infiltration is associated with a good prognosis in epithelial neoplasms. Intratumoral T cells correlate with delayed recurrence or delayed death in multivariate analysis in ovarian carcinoma,[24] small cell lung cancer,[52] esophageal cancer,[27] and renal cancer[53] and may be associated with increased expression of specific cytokines and lymphocyte-attracting chemokines within the tumor.[24] The absence of intratumoral T cells is associated with increased levels of angiogenic factors

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such as vascular endothelial growth factor (VEGF) in ovarian cancer.[24] The characterization of the frequency of T-cell subsets in tumors may warrant special attention. Usually, CD4$^+$ and CD8$^+$ cells are both present, but their ratio in tumors may differ from peripheral blood. In ovarian cancer primary sites, the two subsets correlate significantly, but CD4$^+$ cells are more frequent.[24] In colorectal cancer, a low CD4$^+$:CD8$^+$ ratio predicts significantly higher 5-year survival, independently of Dukes stage and age.[47]

T-Cell Subsets

Detailed characterization of T-cell subsets in naïve, central memory (T_{cm}), and effector/memory (T_{ef}) subsets based on cytokine profile, immunophenotype, and chemokine receptors is now possible,[54,55] but it remains experimental as it necessitates multicolor flow cytometry. CD4$^+$CD25$^+$CD45RO$^+$ regulatory/suppressive T cells (T_{reg}) have recently emerged as an important subset of lymphocytes that mediate peripheral tolerance through suppression of CD8$^+$ function in tumors.[56] The frequency and significance of T_{reg} in cancers remain to be characterized, but specific markers for this T-cell subset remain controversial. Cytotoxic T lymphocyte-associated antigen (CTLA)-4 (CD152) and the transcription factor Foxp3 may be two molecules that warrant investigation in this context.[57,58]

Tetramer Assays

The use of tetramer assays to detect the presence of functional tumor-specific T-cell responses is currently considered an area of research, given the need to HLA type and the paucity of defined tumor rejection antigens in most tumors. Generation of T-cell lines from tumor-infiltrating lymphocytes (TILs) is labor intensive but is another means to assess the number and functional capability of these cells. The presence of HLA class I and II molecules on tumor cells should be assessed in concert with T-cell, NK-cell, and dendritic cell (DC) infiltration. If possible, the assessment of CD3-zeta (CD3ς) chain expression in T cells in various cancer biopsies should be assessed, as its decrease may be associated with an ineffective immune response.[59–64] The clinical significance of CD3ς chain expression in TILs in most cancers remains unclear. Pro-apoptotic signals or anti-inflammatory TGF-β may account for suppression of CD3ς. TGF-β may be produced in large quantities by CD4$^+$CD25$^+$ T_{reg} in ovarian and lung cancer.[65] Thymidine phosphorylase is an angiogenic factor expressed by cancer cells, stromal cells, and tumor-associated macrophages in many human tumors[66–72] and occurs almost exclusively with intense lymphocytic infiltrate. Perhaps assessment of this marker could be used as a surrogate for immune infiltration.

Dendritic cells, Myeloid, and Plasmacytoid

We now recognize at least two different type of tissue DCs: the rather classical myeloid DC, which is the major "professional" antigen-presenting cell, promoting initiation of the adaptive immune response, as well as the plasmacytoid DC, which has a predominantly immunoregulatory role, secreting IFNα and IL-12 in response to immune stimulation. There are now over 100 studies showing DC defects in cancer patients, with increased number associated with an improved prognosis.[73] Increased numbers of DCs, for example, are observed in chronic inflammation[74–79] and correlate with improved prognosis in patients with ovarian cancer,[80–82] colon cancer,[83] mycosis fungoides,[84] renal cancer,[85,86] hepatocellular carcinoma,[87–89] breast cancer,[90–93] esophageal cancer,[94] chronic myelogenous leukemia,[95] pancreatic cancer,[96] and head and neck tumors.[62,97–99] Specific changes have included diminished expression of MHC class II molecules, decreased expression of costimulatory molecules, including CD80 and CD86, and decreased ability to migrate out of tumor sites to regional lymph nodes with stimuli including TNFα in myeloid DCs. Diminished numbers of myeloid DCs, correlating with stage and grade of the neoplasm, have been identified in most adult, but not pediatric, tumors.

Plasmacytoid DCs

A preponderance of plasmacytoid DCs within breast cancer, associated with worst prognosis, has been suggested (Lebeque S, personal communication). An increased ratio of plasmacytoid to myeloid DCs has been suggested as a good finding in the setting of allogeneic transplantation and an adverse finding in pediatric and head and neck neoplasms.[43,100] Plasmacytoid DCs are abundant also in malignant ascites associated with peritoneal carcinomatosis and are attracted by specific chemokines such as SDF-1.[101]

Myeloid DCs

Identification of myeloid DCs in the epithelial compartment of tumors (S-100 or CD1 or DC-SIGN positive) as well as interdigitating reticular DCs (p55 positive) in peritumoral areas has been reported in over 100 separate studies and generally correlates with improved prognosis. CD83$^+$ cells correlate with the presence of intratumoral T cells and improved outcome in ovarian cancer.[80] The number of DCs infiltrating tumor is in general a highly significant prognostic parameter in patients with cancer and should be widely assessed, measured, and reported. Furthermore, the absence or paucity of DCs is strongly linked to abnormalities with the number and phenotype of TILs. Many studies of DCs have assessed CD83$^+$, HLA-DR$^+$, CD40$^+$, and CD86$^+$ expression consistent with activation or a mature DC phenotype. The density of DCs in cancer primaries generally is higher than in metastatic lesions and within primaries typically is associated inversely with grade. Treatment of basal cell carcinomas locally with Toll-like receptor 7 (TLR7) agonists such as imiquimod, stimulating myeloid and plasmacytoid DCs, has been associated with immune infiltrate including macrophages and T cells as well as reduced expression of anti-apoptotic molecules including BCL2.[102] Plasmacytoid DCs infiltrate tumors as well and have been demonstrated, at least in head and neck squamous cell carcinomas, to have diminished ability to produce IFN-alpha in response to CpG motif-containing oligonucleotides. Direct injection of DCs into tumor has been applied successfully in murine[103] and human tumors.[104,105]

NK Cells, B Cells, Other Leukocytes

There is now literature correlating peripheral NK numbers and cytolytic activity in the blood of patients with

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cancer relating to outcome/prognosis. Improved outcome in patients with hepatocellular carcinoma,[106–109] gastric carcinoma,[110,111] colorectal cancer,[31,112,113] and multiple myeloma,[114] has been associated with enhanced NK activity and number in the peripheral blood as well as the tumor. NK cells mediate cytotoxic activity against tumor cells, serving as a rapid means to sample stressed cells in infection and cancer.[115,116] They have been difficult to identify in various tumor types but have been found using CD56 and CD57 staining in some human carcinoma-infiltrating lymphocytes and to be associated with a favorable tumor outcome. Intratumoral infiltration of NK cells could be important as a variable with prognostic value, especially in patients with early disease. **CD20+ B cells** have been observed in some malignancies. In tongue lesions, an increase in B lymphocytes correlating with transformation level ($P < 0.001$) has been observed.[117] B lymphocytes are also the predominant lymphocyte in premalignant cervical lesions.[118] A high proportion of terminally differentiated oligoclonal plasma cells expressing presumably tumor-specific immunoglobulin (Ig) are found in the tongue, in the cervix, and medullary carcinoma of the breast (MCB). MCB has a more favorable prognosis than other types of breast cancer at similar stages of differentiation.[119–121] This improved clinical outcome is associated with the presence of a prominent lymphoplasmacytic cell infiltrate, with oligoclonal tumor-infiltrating B-cells in the tumor stroma, some of them autoimmune, responding to tumor actin fragments. **Peritumoral mast cells** were associated with diminished local and distant recurrence (44% vs. 15%, $P = 0.007$ and 86% vs. 21%, $P < 0.0001$, respectively) and improved survival in patients with rectal carcinoma.[122] The presence of intratumoral T cells independently predicted diminished occurrence of distant metastases (32% vs. 76%, $P < 0.0001$). It has been suggested that mast cells may regulate lymphangiogenesis in colorectal carcinoma, in melanoma,[123] and in Hodgkin's disease,[124–127] where they are the predominant cell expressing CD30L.[124,125]

Presence of Necrosis

In most tumor types, a particularly bad prognosis is associated with tumor necrosis. This has been found in non-small cell lung cancer,[128,129] breast cancer,[130–134] melanoma,[130,135,136] sarcoma,[137–140] colorectal carcinoma,[141,142] and non–clear cell renal carcinoma.[143–145] This had been attributed to rapid cell proliferation associated with inadequate or unavailable blood supply. This assessment, if routinely included and graded in all pathology reports, would enhance the value of retrospective analyses evaluating and subsetting human tumors. Necrosis has been defined by morphologic criteria, but due to massive degradation, reliable molecular markers have been elusive. Recently, high mobility group box chromosomal protein 1 (HMGB1) has been described as a marker of necrotic death,[146–149] acting as a cytokine within the cell. Its application in histopathology of tumors remains to be defined.[150,151]

What Should Be Applied Now?

Perhaps the strongest recommendations/results that this group could provide is that an assessment of immune cells (T/NK/DC/B; neutrophils, eosinophils, and mast cells) and enumeration in all pathologic evaluations of tumors in humans should be carried out and should/could be considered state of the art. In addition, quantitative assessment of micro- and macronecrosis should be evaluated in all tumors.

Ways to Facilitate Advances

The data coming from assessment of immune cells (see below) and the presence of necrosis in tumors and their relationship to prognosis are so compelling that modern pathologic reports of tumors should reflect this. This needs to be aggressively developed and promoted. In the future it may be used in part as a means to subset tumors being evaluated by modern genomic/proteomic strategies. We are most interested in having pathologists identify both immune and nonimmune cells in the tumor, perhaps correlating with the findings in the peripheral blood that predict response to immunologic treatments. Efforts should concentrate on standardization and quality control. Evaluation of individual cell types and their correlation with each other and with outcome/other modern techniques should be carried out. The inverse relationship of tumor infiltrate with tumor necrosis (see above), a particularly adverse prognostic finding, should be explored. This needs to be communicated and developed with experts and thought leaders in pathology academic centers and laboratories.

Practical Issues/Recommendations to Advance This Most Quickly

Surveys of tumors conducted post hoc or in limited single-institution trials have suggested the utility of several assays in defining patient prognosis or outcome. Their utility in defining recurrence, measuring disease, and in concert with other measures defining a strictly correlative surrogate has not been clearly communicated or made standard practice. We would recommend that all patients with a diagnosis of tumor have immune infiltrate graded and specified (T, B, NK, pDC, mDC, mast cells) and that necrosis be quantified using available microstaging systems. Furthermore, we would recommend that all patients in federally funded studies have peripheral blood mononuclear cells banked for future assessment of baseline NK/T/DC phenotype and function. Furthermore, in the context of biotechnology and pharmaceutical-sponsored neoadjuvant, adjuvant, and therapeutic strategies, PBMCs should be assessed sequentially for NK cytolytic activity, DC phenotype and subsetting, and T-cell immunoscope analysis. A central group reviewing and assessing these data in the context of ongoing clinical trials should convene and assess advances/insights arising from this analysis on a regular basis. Federal funding mechanisms should solicit validation of studies of the above markers in the context of large-scale prospective or retrospective studies.

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SUMMARY AND FINDINGS OF THE WORKSHOP

As different array platforms, experimental designs, material preparations, and analysis tools are employed more widely, data comparison becomes a daunting and often frustrating task. Not only the comparison but also the validation of assays identified as biologic markers and in particular as surrogate markers are often viewed skeptically. The demand for standardization of these types of data is rapidly increasing as large databases accumulate. We would recommend the following be implemented and validated/confirmed by other groups active in this area, including the American Association of Cancer Research, the American Society of Clinical Oncology, and the Society of Surgical Oncology:

1. Minimum Information About a Microarray Experiment (MIAME) should be used (see above) to standardize array data and metadata presentation.
2. In the process of developing high-throughput technologies, promote assay standardization.
3. Promote automation technologies that will reduce the amount of sample manipulation, thus reducing interoperator variability and producing more consistent analyses between individual clinical laboratories.
4. NIH/FDA/Biotech Cooperative Grants (non-SBIR) be funded to accelerate clinical applications.
5. NIH/FDA/Pharmaceutical companies should provide training/upgrades to staff involved in applying these new technologies.
6. Develop core facilities with an array of advanced instruments/technologies available.
7. Enable sharing of results with development of open-access multi-institutional website, similar to NCI WEB site.
8. Develop continuous upgrades of bioinformatics tools.
9. Implement training and subsequent testing systems on assays (normal vs. disease) with large databases.
10. As each new patient is validated through pathologic diagnosis using retrospective or prospective data, add its input to the expanding training set.
11. Establish a database of normal ranges from various demographic populations to allow valid comparisons to disease states.
12. Create a national repository for serum/plasma as well as definition of the best practices to use for serum/plasma collection.
13. Standardize serum/plasma analysis, storage, and good laboratory practice through a combined effort of interested groups, including the NCI, FDA, WHO, Red Cross, etc.
14. Fund and perform a definitive study of selected immune response gene polymorphisms in selected cancers, such as cutaneous malignant melanoma, breast cancer, and/or childhood leukaemia.
15. Insist that all patients in biotherapy studies have assessed at baseline and following therapy T-cell counts (total, CD4, CD8) and T cell receptor ζ-chain expression by flow cytometry.
16. Develop integrated strategies to enhance antigen detection with immunohistochemistry strategies.
17. Coordinate with academic pathology groups to make standard of practice tumor biopsy assessment of intratumoral immune cells (T/NK/DC/B; neutrophils, eosinophils, and mast cells) and enumeration in all pathologic evaluations of all tumors in humans of micro- and macronecrosis. The iSBTc will review these recommendations internally with its membership and promote their implementation in the appropriate forums.

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