The identification of clinically relevant markers and therapeutic targets

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The history of tumor biomarker discovery has been one of limited success. Population based screens are few and of limited clinical usefulness. Biomarkers that are able to segregate patients by diagnosis, prognosis and appropriate therapeutic selection are in great need and will be the basis of the clinical management in the future. This review sets out the challenges inherent in the field of tumor biomarker discovery and the tools that we are using to meet that challenge. It is now possible, using this suite of technologies, to discuss novel tumor biomarkers in terms of a pipeline rather than single unique events in research. The future of clinical oncology management will use markers such as those being identified via these techniques to improve patient care through better diagnosis and hopefully to achieve greater success in treatment by exploiting tumor markers as therapeutic targets.

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▼ The identification, development and validation of tumor biomarkers have challenged the research and development community for over 150 years. Although there have been some successes, a biomarker that is useful in the early diagnosis of disease in otherwise asymptomatic individuals has proven to be an elusive goal. The majority of the existing tumor biomarkers are most useful in educating a clinical decision-making process after an initial suspicion has been raised by more conventional means. The Human Genome Project presages, among other things, a new age of tumor biomarker discovery. In this new era, genetic and genomic information will be mined for markers useful to the five main categories of tumor biomarker use: screening, diagnosis, prognosis, therapeutic monitoring and prediction of disease recurrence. This promise is in its earliest stages of realization. The availability of the draft sequence of the human genome does not change the basic premise for all of tumor biomarker discovery, which is the need for markers with high sensitivity, specificity and predictive value.

Historically, the process of tumor biomarker discovery has been one of brute force identification or serendipitous discovery. Individual tumor markers were often identified as the result of a laborious series of experiments isolating individual proteins followed by testing a small group of patients for the presence of that protein. Despite this significant amount of work the markers that resulted were often of limited use in broader clinical populations, particularly as screening tools in populations where the prevalence of disease is relatively low. The narrower uses of tumor biomarkers as adjuncts to diagnosis, prognosis, monitoring and recurrence can claim more success but the overall number of markers remains limited. Table 1 lists some of the most commonly used markers and their indications.

As can be seen in Table 1, the number of clinically useful screening tools is limited. In addition, the vast majority of tumor markers can not be used as primary diagnostic tools because of their lack of appropriate specificity and sensitivity when used in the general population [2]. It is likely that the next generation of tumor biomarkers will follow a similar pattern of identification and development. The demands placed on the development of a useful screening tool with respect to sensitivity and specificity in a general population where incidence of disease is low are often too great to be overcome by a single biomarker. This does not, however, alter the potential for biomarker clinical relevance when used in conjunction with clinical findings for purposes outside of screening. The challenge for the next generation of biomarkers will be to take advantage of the increasing knowledge concerning disease pathways. As therapeutic development efforts focus more on the molecular pathology of disease and develops

1359-6446/02/$ – see front matter PII: S1359-6446(02)02543-6

Table 1. Commonly used serum tumor markers

Tumor marker[a]	Malignancy	Primary application	Non-neoplastic conditions
Alpha1 fetoprotein	Hepatocellular, germ cell tumors (testis)	Diagnosis, monitoring	Cirrhosis, hepatitis
CA125	Ovarian	Monitoring, prognosis	Menstruation, peritonitis, pregnancy
CA15-3 (CA27-29 similar profile)	Breast	Monitoring	
CA19-9	Pancreatic	Monitoring	Pancreatitis, ulcerative colitis
Carcinoembryonic antigen	Gastrointestinal, breast, other adenocarcinomas	Monitoring	Pancreatitis, hepatitis, inflammatory bowel disease, smoking
Human chorionic gonadotropin	Nonseminomatous germ cell tumor (testis) choriocarcinoma, seminoma, mola hydatidosa	Diagnosis, monitoring, prognosis	Pregnancy
Prostate specific antigen	Prostate	Screening, diagnosis, monitoring	Prostatitis, benign prostatic hypertrophy

[a]Adapted from Ref. [1].

therapies keyed to specific disease processes, it will be necessary to identify those patients with specific molecular abnormalities before the selection of appropriate treatment(s). The development of technologies that take advantage of the response that individual patients mount to the challenge of disease will enable the identification of clinically relevant biomarkers in a more rapid manner. Coupled with the development of molecular therapeutics, this will enhance the average therapeutic response by focusing on the patient's specific tumor biology. The immediate need for appropriate tumor markers that would enable early detection of disease is underscored by the general consensus that, with early intervention, even existing treatments that work poorly in advanced disease could be effective.

Tumor biomarker properties

To better understand why the great majority of once promising tumor markers have failed, we review the properties that a useful marker must possess and the reasons why a marker's use could be mitigated. An ideal tumor marker would have 100% sensitivity (i.e. no false negatives) and 100% specificity (i.e. no false positives). Such a tumor marker would lend itself to the full range of uses. To qualify for any diagnostic application, the test for the marker would require sufficient sensitivity and favorable assay variability. For screening purposes, where the most stringent criteria is required, the use of tumor markers that do not achieve 100% specificity or sensitivity are dependent on other factors. The most significant factor, is the prevalence of the disease in the population tested [2–3]. The dependence of marker use on disease prevalence can be illustrated by considering the composite statistic known as positive predictive value (PPV; based on Bayes theorem). PPV is calculated according to the following equation [Eqn 1] :

$$\mathrm{PPV} = \frac{(s)(p)}{(s)(p)+(1-sp)(1-p)} \qquad \text{[Eqn 1]}$$

This measure of biomarker use represents the percentage of marker-positive patients in the tested population that have cancer. Note that when sensitivity (*s*) and specificity (*sp*) are 100%, the PPV is 100%. But when *sp* is less than 100%, low population prevalence (*p*) tends to decrease the PPV and, therefore, the use of the marker as a screening tool because of the resulting increase in the number of false-positives that will be detected. The dependence of PPV on disease prevalence is illustrated in Fig. 1. Note that for illustrative purposes, PPV has been calculated at various fixed *s* and *sp* values ranging from 50% to 90%.

Markers suitable for disease screening have been most elusive because of the requirement for exquisite sensitivity and specificity. It is primarily for this reason and the reason that marker elevation is frequently not seen in early disease, that all markers studied to date have failed as screening tools for the general asymptomatic population. The problem is further compounded by the fact that the known tumor markers are frequently elevated in individuals with benign neoplasias [4–9]. In a diagnostic rather than screening application, one can increase disease prevalence (and therefore PPV) by testing highly selected populations with increased disease risk. However, this approach tends to decrease the specificity of the marker by increasing the detection of marker-positive individuals as a result of the increase in persons with benign neoplasias, as has been the case with the known tumor markers. Likewise, attempts to use multiple markers for screening or diagnosis tend to increase sensitivity, but at the expense of decreased

specificity [10–11]. Therefore, these methods to increase PPV reach a point of diminishing return when considering screening applications and highlight the need for new tumor markers with inherently higher sensitivity and specificity.

Biomarker discovery: opportunities and challenges

Although few validated markers have been identified to date, despite a worldwide effort spanning at least five decades, many more potentially useful markers are being discovered at an accelerated pace. The driving force behind this has been the development of advanced genomic and proteomic methodologies directed at identifying those genes expressed at elevated levels in tumors compared to normal tissues. Genomics-based techniques currently employed include Serial Analysis of Gene Expression (SAGE) [12], microarrays [13], subtractive hybridization [14], as well as analysis of microsatellite instability [15], DNA methylation patterns [16], and nucleotide polymorphisms [17]. Proteomics tools currently being used include MS techniques [18], high-resolution 2D electrophoresis [19], and antibody and protein arrays [20–21]. Collectively, these methods have the potential to produce tumor markers with significant screening, diagnostic and therapeutic value.

Most known tumor markers have been identified by analyzing gene expression patterns in tumor cells or proteins that are present in body fluids of cancer patients. These methods have identified gene products that are expressed either in supernormal quantities or exclusively by tumor cells as compared to normal tissues or at elevated levels in patient body fluids. These approaches have yielded tumor-associated genes that suffer from a lack of sufficient specificity and/or sensitivity, compromizing their use as reliable screening, diagnostic or prognostic tools. This is because tumors frequently employ natural physiologic mechanisms for proliferation, invasion of surrounding tissue and metastasis that are also employed by normal cells throughout the body. Indeed, most potential tumor markers identified to date are normal non-mutated cellular proteins that, although overexpressed in tumors, are also expressed to some degree in benign neoplasias as well as some normal tissues. Here we review several of the genomics-based marker identification technologies that we believe hold promise for identifying superior tumor markers suitable for screening and diagnostic, as well as therapeutic applications.

Microarrays

Microarray technology enables the simultaneous interrogation of the expression level of thousands of genes to obtain a quantitative assessment of the differential abundance of nucleic acids in a given tissue or cell. The implementation of large-scale genetic profiling has expanded the definition of biomarkers to include a set (sometimes a large set) of genes whose expression is important in the differential diagnosis of a particular disease. A prime example of how this can be used to improve clinical practice is the work of Yeoh *et al.* [22] on the classification of pediatric acute lymphoblastic leukemia (ALL). Through the use of microarrays containing probes for over 12,600 genes the expression profile of a large number of pediatric ALL cases were determined. By the subsequent classification of expression profiles into subtypes the investigators were able to identify seven different classes of pediatric ALL, including a previously unknown subtype. In addition to the diagnostic use of this work, it was determined that expression profiles measured at the time of diagnosis could be useful in the prediction of which patient was most likely to relapse following standard chemotherapy. A unique application of this technology was the determination of a separate expression profile identifying a subset of patients who will respond to the standard chemotherapy for ALL but will be subsequently diagnosed with a secondary acute myelogenous leukemia. This work provides some of the most compelling evidence to date that gene expression profiles could be used for differential diagnosis, prognosis, therapeutic selection and predisposition. Although the use of an expression

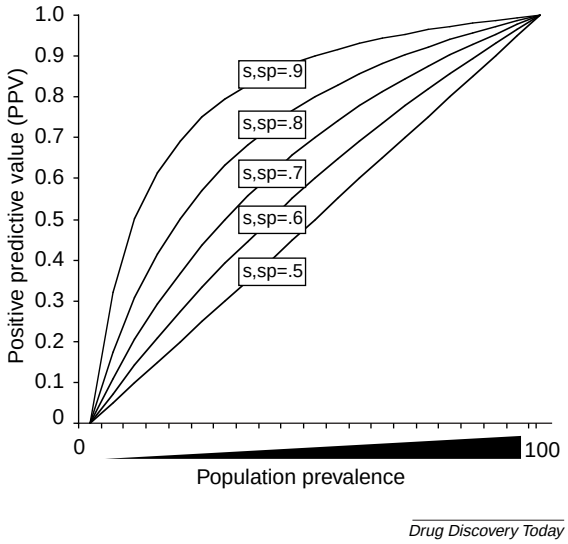

Figure 1. Relationship between population prevalence and Positive Predictive Value (PPV). These plots were generated by setting sensitivity (s) and specificity (sp) in Equation 1 to arbitrary values ranging from 50–90% (i.e. 0.5–0.9) as indicated on the graph. These data illustrate that low population prevalence depresses the PPV, which directly impacts the use of markers as screening tools, even those with relatively high s and sp values.

profile comprised of tens to hundreds of genes is a departure from the current form of biomarker consisting of a single analyte it could, however, be one of the future stalwarts of biomarker analysis.

SAGE

SAGE is a powerful genetic profiling technology that can be applied to any cell line or tissue. SAGE analysis not only enables a qualitative assessment, but also a highly quantitative assessment of gene transcript populations; the data collected from the specimen includes all transcribed genes and the relative number of copies of each transcript. Through the use of multiple libraries and appropriate pair-wise comparisons it is possible to identify expression patterns and sets of genes that could be useful as tumor biomarkers. SAGE has been used to identify potential tumor biomarkers in a wide variety of organ systems [23–27].

The work of Nacht *et al.* [28] demonstrates how SAGE can be used to identify gene expression patterns useful in the differential diagnosis of lung cancer. By analyzing a relatively small series of non-small cell lung carcinomas (NSCLCs) and normal lung epithelium in SAGE experiments it was possible to identify distinct expression patterns. Not only were the NSCLCs different from the normal lung tissue but adenocarcinomas were found to have a distinct expression pattern from squamous carcinomas. When the expression patterns were tested using a larger number of tumors, the patterns were found to be consistent across individuals. Comparing SAGE data with expression arrays demonstrated two important points. First, SAGE data derived from a relatively small set of tumor samples can provide reproducible expression patterns when compared to higher throughput methods. Second, several of the genes found to be part of an expression pattern were not available on the arrays used for higher throughput testing limiting and potentially biasing the data derived from those arrays. The latter point in particular highlights the comprehensive and unbiased nature of SAGE data. This straightforward approach to gene expression pattern identification is most useful when there are relatively pure populations of the particular cell types in question or when relatively gross differences between malignant and normal are present. A more selective approach to gene expression differences can be seen in the next example of SAGE analysis.

The work of St. Croix *et al.* [29] demonstrates how SAGE can be used to identify tumor-specific markers when a specific cell population is isolated. The identification of markers specific to tumor vasculature is a significant advance in the study of tumor angiogenesis. To study the expression differences between tumor and normal endothelial cells that line blood vessels, St. Croix painstakingly isolated endothelial cells through a series of antibody based positive and negative selection steps. The resulting pure population of endothelial cells was then subjected to SAGE analysis and comparison. A series of genes were identified demonstrating either over- or underexpression in tumor endothelium, compared with normal expression levels. As described by St. Croix *et al.* [29], some of these genes had been previously identified and characterized but others, the tumor endothelial markers (TEMs) were unknown as genes before that analysis. By comparing expression levels between highly selected populations of cells it was possible to identify markers of angiogenesis that are important to tumor growth, wound healing and normal vessel growth. Work to characterize these genes further is under way with the goal of using these genes as potential therapeutic agents and markers.

St. Croix's work demonstrates the power of SAGE analysis when applied to the specific isolation of cell types. Another example of the usefulness of SAGE demonstrates how sampling a primary tumor and metastatic growth can identify differential expression profiles important to the spread of malignant disease. Saha *et al.* [30] performed a SAGE analysis using malignant colorectal tumors, benign colorectal tumors, normal colonic epithelium and metastatic tumors obtained from the liver. Following an immunoaffinity purification procedure to specifically isolate metastatic colon tumor cells from hepatic cells, SAGE analysis revealed a series of genes that were up- or down-regulated specifically in metastatic growth. One of the most interesting of these genes was protein tyrosine phosphatase type IVa (PRL-3). PRL-3 was found to be upregulated in all metastases studied but in none of the nonmetastatic tumors or normal colonic epithelium. In several of the metastatic tumors, PRL-3 was amplified at its locus of 8q24.3 indicating a potentially important role for this gene in metastatic tumor development. Therefore, the PRL-3 gene has the potential to serve as a therapeutic, as well as a disease recurrence, marker.

SAGE analysis is an unbiased, sensitive, comprehensive gene expression technology. It provides extensive information on both known and previously unidentified genes resulting from any relevant pair-wise comparison. Gene expression patterns identified through methods such as SAGE will provide novel individual biomarkers in the future as well as contributing to the changing definition of biomarkers, as patterns of expression are further elucidated.

CNAPS

The use of circulating nucleic acids isolated from plasma or serum (often referred to as CNAPS) is conceptually changing

how nucleic acid based biomarker testing is considered. This approach illustrates how the definition of what constitutes a biomarker is likely to change in the near future. Although the concept of CNAPS has been in the literature for some time, it is only relatively recently that applications in several disparate areas have started to appear.

In 1989 Stroun *et al.* [31] were the first to clearly demonstrate that tumor derived DNA could be extracted from plasma. Five years later, two papers demonstrated the presence of various forms of the ras oncogene family in specific types of malignancy [32,33]. The ability to identify tumor specific markers in serum or plasma and use those markers for diagnosis, prognosis and disease recurrence testing is a significant advance in biomarker development. The 2nd *International Symposium on Circulating Nucleic Acids in Plasma and Serum* held in Hong Kong in 2001 presented a series of papers on the use of CNAPS as a sample source providing information on the methylation status, loss of heterozygosity and tumor-associated viral presence in patients afflicted with a variety of tumor types [34]. It is still early in this avenue of investigation; however, the accomplishments to date are clear indications of the value of CNAPS in providing clinically relevant prognostic and treatment selection information. As pathways of disease progression are identified the genes involved in those pathways will become additional potential templates for analysis in this manner.

SEREX

Another powerful technology that enables the exploitation of a patient's B cell repertoire to identify tumor markers is SERological analysis of Recombinant cDNA Expression libraries (SEREX) [35]. This approach involves screening serum antibodies for reactivity with bacteriophage plaques representing an autologous tumor expression library.

Many antibody targets have been identified via SEREX, including previously known tumor-associated proteins, such as MAGE-1 and tyrosinase, as well as novel antigens such as NY-ESO-1. The Ludwig Institute for Cancer Research maintains a publicly accessible database containing results obtained using SEREX from a worldwide network of researchers (http://www.licr.org/SEREX.html). Several of the antigens discovered by this method are the subject of recent or ongoing immunotherapy clinical trials and it remains to be seen whether any of the identified antigens will be suitable for screening, diagnostic or therapeutic purposes.

Cancer antigen discovery: following biology's lead

The search for useful tumor markers by any technology platform is predicated on the idea that common biological alterations are shared among independently-derived tumors and that the identification of the genes or proteins expressed during tumorigenesis could betray the presence of disease at the molecular level, even before clinical manifestation of symptoms. This implies that at least a subset of genes modulated during the development and progression of cancer is not the result of completely random events. Therefore, biological responses (e.g. immune responses) to altered gene expression might also be shared and could provide an opportunity to detect mutant or altered gene expression in tumors. Additionally, the immune response itself to particular antigens could have diagnostic and/or prognostic value.

The idea of exploiting immunosurveillance to identify tumor markers has been postulated previously [36–39]; however, techniques to rapidly identify the relevant cellular immune targets have, even today, remained difficult. Although SEREX technology is an important advance in exploiting the humoral immune response against tumors to identify tumor antigens, we propose that the natural surveillance provided by the cellular immune system could be harnessed to identify those genes (i.e. antigens) that are differentially expressed as a result of tumorigenesis. To fully exploit this approach, high throughput antigen discovery technologies must be developed to achieve comprehensive antigen profiling in cancer patients directed at defining the 'cancer immunome' (i.e. the subset of the proteome that is immunogenic).

The cellular immune system has evolved specifically to detect and eliminate intracellular pathogens. The mechanism by which this is achieved also lends itself to the detection of changes in endogenous gene expression patterns. This immune response against self-antigens, whether mutated or aberrantly expressed, is most efficiently mobilized against those that are minimally expressed in normal tissues. Nearly all resected tumors have been shown to be infiltrated with immune effector cells such as antigen-specific MHC class I-restricted cytotoxic T cells (CTL) [40,41]. Our approach is to clone these immune effector cells directly from freshly resected tumors and employ a functional genomics approach to rapidly identify the antigen specificity of the clones. In addition to being candidate screening, diagnostic and prognostic reagents, these antigens might represent superior immunotherapeutics because, by definition, they are immunogenic. Those antigens that are capable of establishing an immune response driven by the presence of tumor could represent antigens with the lowest tolerance barriers and, therefore, the lowest hurdle to generating a potent immune response under optimal vaccination conditions.

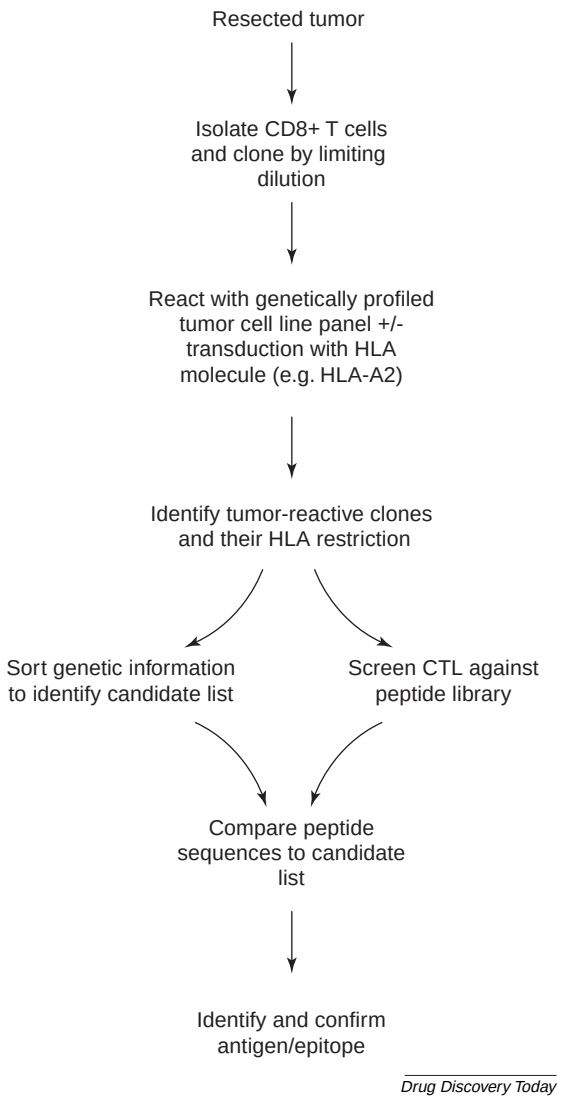

Figure 2. Exploiting natural immune responses to identify tumor markers. The flow diagram illustrates a proposed approach to identifying tumor markers based on the presence of cytotoxic T cells (CTL) that infiltrate most tumors. For any given T cell cloned from a resected tumor, our method leads to the identification of (1) the gene encoding the antigen, (2) the native epitope processed and presented by the tumor and (3) reactive altered peptide ligands that might find use as vaccine components themselves (see text for details).

Identification of T cell targets

Figure 2 illustrates our high throughput method to identify cytotoxic T cell (CTL) targets. We propose that a hypothetical solid tumor is resected for analysis. Standard techniques can be employed to isolate the infiltrating $CD8^+$ cytotoxic T cells from the tumor microenvironment and clone them by limiting dilution. These cells can be driven to proliferation without the need for cognate antigen by using an anti-CD3 antibody and cytokines (e.g. IL-2). Generally, we find that it is possible to generate several hundred clones from a typical tumor specimen. After the clones have achieved a sufficient density (~6 weeks) they can be characterized further for their ability to lyse allogeneic, HLA-matched tumor cell lines. This analysis can be used to determine the clones of greatest interest (i.e. tumor-specific) as well as to elucidate the HLA-restriction of the clones. For example, if each clone is tested for reactivity with a panel of tumor cell lines that have been genetically modified to express a particular MHC allele (e.g. HLA-A2), any naturally $HLA\text{-}A2^-$ cell line that is lysed only when made to express HLA-A2, will indicate that the T cell clone is recognizing a tumor-associated antigen that harbors an HLA-A2-restricted epitope that, by definition, is naturally immunogenic.

The next step in identifying the antigens of interest is to analyze the transcriptomes of the tumor target cells that were employed. We have chosen to genetically profile our tumor targets using SAGE because the data can be sorted in such a way to generate a list of candidate genes that could encode the antigen of interest. For example, if a T cell clone lyses 4 out of 8 tumor cell lines, one could generate a list of those genes expressed above a selected threshold in the lysed lines that are also expressed below a selected threshold in the cell lines that are not lysed. This method can result in lists of less than 50 candidates. Also, note that the SAGE data, once it is generated, can be reused for any future analyses. Therefore, after the initial 6-week expansion phase for the clones, a relatively small solution set of candidate antigens can be generated within one day. Furthermore, hundreds of clones can be simultaneously analyzed by this method.

To define the antigens corresponding to T cell activities of interest, additional information is required. We have developed a method by which a combinatorial peptide library of nearly 50 million species can rapidly be screened for those species that react with the clone under study [42]. This method, Solid-Phase Epitope REcovery (SPHERE), can be completed in less that 2 weeks and generates reactive peptide sequences, some of which are homologous to the native epitope. These peptides can be used to identify which of the genes on the candidate list generated by the SAGE data are the actual antigen. This is achieved by using computer algorithms to align each of the SPHERE peptides with the candidate proteins predicted by the SAGE analysis. Confirmation that the correct antigen is identified is accomplished by synthesizing the native epitope and demonstrating that the corresponding T cell clone interacts with it.

Collectively, these methods represent a new functional genomics approach to the high-throughput identification

of tumor markers and potential immunotherapeutics with the immune system acting as 'filter' for relevant expressed genes.

The future of biomarkers in medicine

To discover biomarkers of practical value to the healthcare system a wide variety of technologies will be required. The combining of biomarkers to increase their use in diagnosis, prognosis and therapeutic selection will become more common and the types of samples able to be tested will expand. As our understanding of the molecular basis of disease grows, the definition of what constitutes a biomarker will inevitably change.

The ultimate value of tumor biomarkers will lie in their ability to inform clinical decisions and positively affect patient care. The criteria applied to the selection of biomarker candidates will need to recognize the limited availability of health care dollars and the need for tests of real clinical value. These criteria will have little to do with the traditional use of markers as tools for population screens. The most extensive use of biomarkers in the future will be post-screening and pre-treatment. The development of this next generation of biomarkers will require answers to several important questions. How is clinical practice changed as a result of this new biomarker? Can a therapeutic course of action be determined from the information provided by this biomarker? Does the presence or absence of the biomarker indicate the success or failure of a particular therapeutic modality? Is additional information available when the biomarker is used in combination with other biomarkers? What, if anything, does the biomarker indicate about the mechanism of disease formation or progression? Answers to questions such as the above will be necessary to build a consensus for biomarker use as well as provide evidence that the knowledge gained is worth the cost to the healthcare system.

Biomarkers hold a significant place in the medical testing armamentarium. The output of the Human Genome Project will increase the opportunities for the biomarker development community to identify disease pathways and determine markers of clinical response. What will not change is the effort required to bring a screening test to full clinical use. The population studies and clinical correlates that are required of a screening test take years of prospective study before reliable conclusions can be reached. Tests for diagnostic, prognostic and treatment selection, however, are more amenable to clinical trial analysis bringing them to routine use in a relatively rapid manner. Much of the next generation of therapeutic development will be based on specific molecular targets. This coming era of molecular pathology will change how disease is classified and treated. The administration of appropriate therapy based on specific molecular targets will require definitive knowledge as to the molecular basis of an individual's disease. This change will bring pathologists and their collection of biomarker tests much closer to the therapeutic decision-making process. The diagnosis and treatment of cancer is changing for the better and biomarkers and molecular pathology will lead the way. In the past, brute force technology has guided the process of tumor biomarker identification. Letting biology be the guide will produce better and greater numbers of clinically relevant tumor biomarkers for the molecular medicine of the future.

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